STUDIES RELATED TO PORTAL HYPERTENSION

By

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DECLARATION OF ORIGINALITY

I declare that the work presented herein and

the composition of this thesis is my own.

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LIST OF ABBREVIATIONS

AVP	Arginine vasopressin
cGMP	cyclic GMP
CI	Confidence interval
CT	Computerized tomography
cm	Centimetres
DHA	Donor hepatic artery
D-NNA	N-nitro-L-arginine (optical isomer of L-NNA)
ELISA	Enzyme linked immunosorbant assay
eNOS	endothelial Nitric oxide synthase
FHVP	Free hepatic venous pressure
G/I ratio	Glucose/insulin ratio
GI	Gastrointestinal
gm	Gram
GOV	Gastro-oesophageal varices
Hg	Mercury
HCC	Hepatocellular carcinoma
I/G ratio	Insulin/glucose ratio
IGT	Impaired glucose tolerance
iNos	inducible Nitric oxide synthase
IVC	Inferior vena cava
KHS	Krebs'-Henseleit solution
L/S ratio	Liver/spleen uptake ratio

L-NAME	N-nitro-L-arginine (methyl ester)
L-NNA	N-nitro-L-arginine
M Bq	Mega Bacqurel
μmol	micromol
min	Minutes
mls	Millilitres
mm	Millimetres
mM	Millimoles
M	Molar
M.C.	Maximal contraction
MHz	Megahertz
MRI	Magnetic resonance imaging
NIDDM	Non-insulin dependant diabetes mellitus
nmoles	Nanomoles
NO	Nitric oxide
NOS	Nitric oxide synthase
OGTT	Oral glucose tolerance test
OLT	Orthotopic liver transplantation
PE	Phenylephrine
pmol/l	Picomoles/litre
PPG	Portal pressure gradient
PSI	Primary shunt insufficiency
RAS	Renin-angiotensin system
R-E cell	Reticulo-endothelial cell

RHA	Recipient hepatic artery
ROI	Region of interest
SD	Standard Deviation
s.e.m.	Standard error of the mean
SI	Shunt insufficiency
SLTU	Scottish liver transplant unit
SPECT	Single photon emission computerized tomography
TIPSS	Transjugular intrahepatic porto-systemic stent shunt
TNF-α	Tumour necrosis factor-α
UWS	University of Wisconsin solution
vs	Versus
WBC	White blood cell
WHVP	Wedged hepatic venous pressure

SECTION 1

INTRODUCTION

INTRODUCTION TO THE THESIS

Cirrhosis of the liver is a chronic disorder resulting from a variety of known and unknown aetiological factors, which lead to hepatocyte damage and death, regeneration of remaining hepatocytes and progressive fibrosis in a sequential manner. This ultimately leads to distortion of the architecture of the liver lobules. The net result is liver cell dysfunction and an increase in the intrahepatic resistance to blood flow. The latter, combined with alteration of portal blood flow and of extrahepatic splanchnic vascular responsiveness characterizes portal hypertension.

Systemic and splanchnic vasodilatation seem to be prominent features of decompensated hepatic cirrhosis and lead to a fall in the effective circulating blood volume. This results in the activation of a cascade of compensatory events, which include the RAS, release of AVP and heightened sympathetic nervous system activity. Neuronal and humoral mechanisms, therefore, seem to be central to the sodium and water retention, which occurs in cirrhosis of the liver. These pathophysiological changes contribute to fluid retention and ascites.

The regulation of arterial tone in cirrhosis of the liver is complex and a multitude of factors seem to be playing a part. The areas which have been explored in the past include the role of endothelial-derived vasodilator agents, circulating vasodilator agents which act on the vascular smooth muscle, and lack of response of the vascular smooth muscle to vasoconstrictor agents.

The last could in fact be a result of the first two factors. There is a paucity of data on human tissues in the literature because of difficulty in acquiring larger distributory arteries from cirrhotic patients for experimentation. Blood vessels acquired at OLT offer the opportunity to study donor (normal) and recipient (cirrhotic) blood vessels *invitro* under controlled standardized conditions (organ bath experiments).

The development of portal hypertension itself is followed by clinically important sequelae. Enlargement of the spleen is an early and cardinal sign of portal hypertension and it is difficult to make a diagnosis of portal hypertension without the demonstration of splenomegaly. Factors which participate in the development of splenomegaly include increase in portal pressure and splanchnic blood flow, and R-E cell hyperplasia. However, the correlation of spleen size to these factors is not entirely clear. The difficulty in interpreting the results of different studies in this area of medicine is partly due to the lack of information about the accuracy of methods used for determining spleen size and partly due to the paucity of data comparing the different methods of measuring spleen size including ultrasonography, CT scanning, MRI and radionuclide studies. Radionuclides have also been used to quantify splanchnic blood flows as well as liver function in the normal and patients with chronic liver disease but there is again a scarcity of data in this regard.

The development of portal hypertension is associated with the opening up of porta-systemic collateral shunts at different sites in order to decompress the portal circulation. Clinically important shunt vessels develop in the oesophagus, stomach and the duodenum. These assume varicose proportions and can rupture when the

intravascular pressures rise above a threshold value. This can lead to potentially life threatening haemorrhage. Shunting of blood to the systemic circulation is also a contributory factor in hepatic encephalopathy.

Impairment of glucose tolerance and even the development of diabetes mellitus are also consequences of cirrhosis and portal hypertension occurring in 10-40% of patients. The prevalence of these abnormalities of glycaemic control increases as the duration and severity of liver disease increases. Although, it is generally believed to be a result of insulin resistance at the peripheral receptor sites, the pattern of insulin secretion from the β cells of the pancreas is also believed to be altered in this form of diabetes, particularly in the later stages. However, there are obvious problems in quantifying insulin secretion from the endocrine pancreas as it undergoes significant extraction during first pass through the normal liver and the pulse mass is lost to a large extent. Measurements done at the peripheral vein site are therefore not an accurate reflection of the quantum of insulin secretion over a period of time. TIPSS used to treat complications of portal hypertension are reviewed at portographic examinations and can be used to study the pattern of insulin secretion directly from the portal vein. Although this complex investigation could only be done in a few patients with relatively stable liver function, this human model can be used to study the pattern of insulin secretion in the patients with different grades of severity of liver disease and normal as well as abnormal glucose tolerance in hepatic cirrhosis.

A number of treatment strategies have been devised for prevention of bleeding and control active bleeding from varices in the gastro-intestinal tract. These include

endoscopic sclerotherapy, endoscopic variceal ligation, pharmacological measures including β adrenoreceptor blocking agents and nitrates, surgical decompressive shunts and TIPSS.

TIPSS is the most recent addition to the armamentarium of treatment modalities. Although it is a highly effective of treatment, there are obvious problems in that procedure related and short and long-term complications limit its acceptability. Procedure related complications include intraperitoneal bleeding and subcapsular haematoma in the liver. The main longer-term problems relate to sepsis, precipitation of hepatic encephalopathy and SI and occlusion. A number of shunt and patient related factors have been assessed as predictors of SI and these include, age and sex of patients, the liver functions, pre- and post-stent insertion portal pressures and the diameter of the stent. The role of diabetes mellitus, which has been shown to encourage stenosis in vascular stents placed elsewhere in the body, has not been assessed in TIPSS.

Accordingly, the aim of this thesis was to explore the following aspects of hepatic cirrhosis and portal hypertension.

AIMS OF THE THESIS

1.1 TO STUDY THE PATHOPHYSIOLOGIC MECHANISM OF VASCULAR HYPORESPONSIVENESS IN HEPATIC CIRRHOSIS.

- The aim in these experiments was to investigate the role of NO release in modulating α-adrenoceptor mediated contraction in hepatic cirrhosis.

1.2 TO STUDY CLINICAL SEQUELAE OF PORTAL HYPERTENSION DUE TO HEPATIC CIRRHOSIS.

These were the areas of study in relation to clinical sequelae:

- The clinical aspects of splenomegaly and its relation to portal haemodynamic factors in portal hypertension due to hepatic cirrhosis.
- The role of radionuclides in measuring spleen size and assessing liver function in hepatic cirrosis and portal hypertension.

1.3 TO STUDY THE PATTERN OF INSULIN SECRETION IN CIRRHOTIC PATIENTS WITH TIPSS AND THE IMPACT OF DIABETES MELLITUS ON TIPSS FUNCTION.

- The pattern of pulsatile insulin secretion in portal vein in patients suffering from hepatic cirrhosis using a TIPSS inserted model was studied. This model should help study pattern of insulin secretion in different stages of hepatogenous diabetes mellitus, ie., impaired glucose tolerance and frank diabetes mellitus.

- The effect of diabetes mellitus on the efficacy of TIPSS in the treatment of patients suffering from portal hypertension has been studied.

ABSTRACT OF THESIS

Cirrhosis of the liver impairs liver function and causes portal hypertension. It leads to many abnormalities including splenomegaly, GOV, portasystemic shunting of blood and widespread circulatory changes. Metabolic changes include diabetes mellitus. TIPSS, a recent treatment for portal hypertension is limited by the frequent occurrence of stent occlusion. I have investigated several of these features of cirrhosis and portal hypertension, and this thesis comprises the results of these investigations:

1: The role of NO as a mediator in the hyporesponsiveness of hepatic arteries in cirrhosis is investigated, *invitro* noting the effect of iNOS inhibition on vascular contractility. **Result:** No difference between the contractile response of cirrhotic and non-cirrhotic blood vessels to phenylephrine was noted with or without iNOS inhibition. **Conclusion:** iNOS activity does not modulate $\propto$-adrenoceptor mediated contractility in hepatic cirrhosis.

2a: To assess the role of clinical palpation in diagnosing splenomegaly in hepatic cirrhosis and to determine the value of abdominal ultrasound in assessing spleen size. Ultrasonography is compared with radionuclide imaging in measuring spleen size. Spleen size has also been related to peripheral blood features of hypersplenism, severity of liver disease, size of GOV and with portal haemodynamic measurements. **Results and conclusions:** Clinical palpation is an insensitive tool in assessing spleen size. There is a good correlation between spleen weight and ultrasonically determined length of spleen. Ultrasonography and radionuclide imaging give

comparable results in measuring spleen size. There is a good correlation of spleen size with portal vein blood flow volume but not with PPG and azygos vein blood flow volume. Spleen size also correlates well with the size of GOV and a history of variceal bleeds. Spleen size also correlated with the peripheral WBC count but not haemoglobin concentration and platelet count indicating that hypersplenism in portal hypertension affects the different formed elements of the blood to different degrees.

2b: SPECT of spleen has been used to assess severity of liver disease in hepatic cirrhosis. **Results:** Spleen uptake of radioisotope per unit volume (% injected dose of radioisotope/ml.) increases and the L/S uptake ratio diminishes as the liver disease worsens. There is also a negative correlation between spleen uptake per unit volume and the size of spleen. **Conclusion:** SPECT of the spleen can be used to assess liver function in patients with liver disease.

3a: To measure the frequency of pulsatile insulin secretion in portal vein of patients suffering from hepatic cirrhosis. The frequency and amplitude of secretory bursts in the portal vein has been compared to that in the systemic circulation in order to see the impact of hepatic extraction and dilution on the peripheral delivery of insulin. The impact of hyperglycaemia on secretory pattern of insulin has also been assessed. **Results and Conclusions:** Direct sampling from the portal vein in human cirrhotic subjects establishes an interpulse interval of ~ 5 minutes. It also shows that the majority of insulin is secreted as bursts and the apparently preferential hepatic extraction of pulses leads to post-hepatic delivery of smaller amount of insulin in discrete bursts.

3b: To assess the role of diabetes mellitus in producing TIPSS insufficiency. **Results and conclusions:** Diabetes mellitus is common in patients undergoing TIPSS and leads to early shunt insufficiency.

SECTION 2

VASCULAR HYPORESPONSIVENESS IN PORTAL HYPERTENSION DUE TO HEPATIC CIRRHOSIS

2.1 INTRODUCTION

Chronic increases in systemic and splanchnic blood flow have been observed in the hyperdynamic circulatory state of portal hypertension in animals and humans. Peripheral vasodilatation seems to initiate the development of decreased systemic vascular resistance and mean arterial pressure, plasma volume expansion, elevated splanchnic blood flow and elevated cardiac index that characterizes this state (Colombato et al, 1992).

The severity of the haemodynamic alterations increases as cirrhosis worsens, with patients with mild disease exhibiting only mild changes and a small increase in plasma volume (Eisenberg, 1956; Ryan et al, 1996). As the disease advances, the circulatory abnormalities, accompanied by increased water retention and increased plasma hormone concentrations become more pronounced (Lieberman and Reynolds, 1967; Willet et al, 1985). These changes may lead to severe complications, including renal failure (hepatorenal syndrome), which is associated with high mortality (Rahman et al, 1992).

Several mechanisms including increased concentrations of circulating vasodilators, increased endothelial production of local vasodilators and decreased vascular responsiveness to endogenous vasoconstrictors have been implicated in the production of peripheral vasodilatation. The last mechanism is likely due to the effect of the first two components. Alternatively, receptor and post receptor defects

in signal transduction could be operative in the impairment of responsiveness to vasoconstrictors in cirrhosis, resulting ultimately in lowering of vascular tone.

2.1.1 Systemic vasodilatation

2.1.1.1 Circulating vasodilators

In portal hypertension, there is an increase in both endothelium-dependent and endothelium-independent vasodilators. The rapid reversal of haemodynamic derangement following liver transplantation (Woods et al, 1987), normal function of transplanted kidneys from cirrhotic donors (Koppel et al, 1969), and the induction of a hyperkinetic state by cirrhotic plasma (Loyke and Hoobler, 1982), strongly implicate a blood borne vasodilator. A number of substances have been implicated as mediators of vasodilatation in cirrhosis of the liver, such as those normally metabolized by the liver and present in abnormal concentrations in advanced liver disease. These include vasoactive intestinal polypeptides (Hunt et al, 1979), prostacyclins (Stitzman et al, 1989), endotoxins (Triger et al, 1978; Lumsden et al, 1988), glucagon (Benoit et al, 1984 ; Lee et al, 1988), and NO (Claria et al, 1992; Stark et al, 1992). There is also evidence of over production of vasodilator substances (Hortnagl et al, 1984; Kravetz et al, 1987). Some substances, normally inactivated or excreted by the lungs, have also been thought to bypass lung tissue in the presence of pulmonary arteriovenous shunts (Rydell et al, 1956; Berthelot et al, 1966; Hansoti et al, 1966). Other mechanisms invoked to account for the greater presence of vasodilator substances in the systemic circulation include intrahepatic (Huet et al, 1982), portacaval (Lebrec et al, 1983) and portopulmonary shunts (Shaldon et al, 1961), these shunts providing bypasses of hepatic tissue.

2.1.1.2 Increased endothelial production of vasodilators: The role of NO.

There is increasing evidence for a major role for the endothelium in the maintenance of basal vascular tone and the development of systemic splanchnic vasodilatation in portal hypertension. The endothelial cells synthesize and release potent vasoactive substances such as prostacyclin, endothelin I, NO, angiotensin II, C-naturetic peptide, platelet activating factor, substance P and histamine (Suga et al, 1992; Casadevall et al, 1993; Ross, 1993). These substances act locally on the vascular smooth muscle cells in a paracrine fashion.

NO which was peviously known as endothelial-derived relaxing factor, is synthesized from L-arginine by the enzyme NOS, which has at least two isoforms: a constitutive (calcium-dependent) form termed eNOS, and an endotoxin- or cytokine-inducible form termed iNOS, in different cell types. This enzyme mediates its potent vasodilatory action on vascular smooth muscle cells through soluble guanylate cyclase.

There is some evidence that elevation of NO synthesis is essential to the development of portal hypertension, and treatment with L-NNA, a competetive inhibitor of NOS, prevented the development of peripheral vasodilatation, decreased systemic arterial pressure, and plasma volume expansion in portal vein ligated rats (Lee et al, 1993). Similarly, chronic administration of L-NAME, another inhibitor of NOS, has been shown to delay splanchnic vasodilatation, development of collaterals and decrease splanchnic blood flow in portal vein ligated rats (Garcia-Pagan et al, 1994). In portal vein ligated rats, isolated aortic rings have also demonstrated

increased relaxation to acetylcholine, an endothelial agonist, compared with sham controls. This increased response to acetylcholine was partly reversed by L-NAME (Karatapanis et al, 1994). Whether the production of NO is the primary stimulus in the development of vasodilatation or a subsequent phenomenon that results from shear, and secondarily contributes to increased flow, has yet to be determined.

It has been hypothesized that the chronic endotoxaemia in portal hypertension and cirrhosis may upregulate iNOS in vascular smooth muscle cells, thus causing the increased NO production that leads to splanchnic vasodilatation (Vallance and Moncada, 1991). The resultant increase in NO release also explains the raised urinary nitrates and nitrites (Bird et al, 1993; Hori et al, 1995) and cGMP levels in cirrhosis (Schneider et al, 1994). Furthermore, direct measurements confirmed that NO concentrations were increased in the splanchnic circulation in cirrhosis (Battista et al, 1997).

TNF-$\propto$ may also play an important role in the vasodilatation by upregulating iNOS. Treatment with anti-TNF-$\propto$ polyclonal antibodies has been shown to ameliorate the hyperdynamic circulation in cirrhosis in animals (Lopez-Talavera et al, 1995, 1996). Induction of iNOS in hepatic cirrhosis has not been proven and the relative contributions of the constitutive and inducible forms of NOS are still under investigation. Data from humans in this area is very limited (Smith et al, 1993; Scheppke et al, 1997). This has prompted the present investigation to be undertaken on hepatic arteries obtained from human subjects suffering from hepatic cirrhosis of advanced stage and undergoing OLT.

2.1.1.3 Decreased responsiveness to vasoconstrictors

Vascular tone is regulated by a complex interaction between endogenous vasodilators and vasoconstrictors. A decreased response to vasoconstrictors may therefore contribute to vasodilatation and thereby to a hyperdynamic circulation. In portal hypertension, *invitro* hyporesponsiveness to the endogenous vasopressors norepinephrine, AVP and angiotensin II has been reported to contribute to the hyperdynamic circulation (Sieber and Groszman, 1992a).

The hyporeactivity of blood vessels to pressor agents appears to be mediated partly by NO. In portal hypertensive rats, inhibition of NO in isolated, perfused superior messenteric artery has been shown to prevent the development of vascular hyporeactivity to the vasoconstrictors nor-epinephrine and AVP (Sieber and Groszman, 1992b).

The situation in human subjects with portal hypertension is less clear. This is partly due to paucity of suitable vascular tissue for *invitro* experimentation and small numbers of subjects being studied in *invivo*. *Invitro* experimentation on tissues provides standardized conditions while eliminating the confounding effect of factors like central and autonomic nervous systems, circulating endogenously produced hormones and intravascular volume mismatches between individuals. Such experiments are possible in centres where OLT is being done and donor (control) and recipient (cirrhotic) blood vessels are available for comparative analysis.

2.2 STUDIES OF *INVITRO* RESPONSIVENESS OF HUMAN HEPATIC ARTERIES IN PATIENTS SUFFERING FROM HEPATIC CIRRHOSIS

2.2.1 Methods to study isolated organ preparations *invitro*

The Organ Bath System

The full system for studies in isolated organ preparations is termed the organ bath system, and has three main elements. The first is an arrangement, which provides standardised, optimal *invitro* conditions for the preperation. This is *the biological system*. It consists of the glass organ bath (see description below and Figure 2.1), the physiological salt solution, thermostatic control (to keep the saline at a constant temperature), provision of oxygen and carbon dioxide aeration of the suspended tissue and physiological salt solution, and the test substance itself. The second is a *measuring device* for recording the change in tension in the smooth muscle (test organ) following the appropriate stimulus. This device comprises of a transducer, amplifier and the recording instrument. The third is the *transmission system,* which forms the mechanical link between the first two components.

The organ bath comprises of a double walled glass chamber in which the test preparation is suspended in physiological saline (Figure 2.1). The saline is maintained at a constant temperature by the flow of water of the correct temperature, through the outer cavity. The water used for this purpose is maintained at a constant temperature (37^0 C in our experiments) with the help of thermostat control in the water bath.

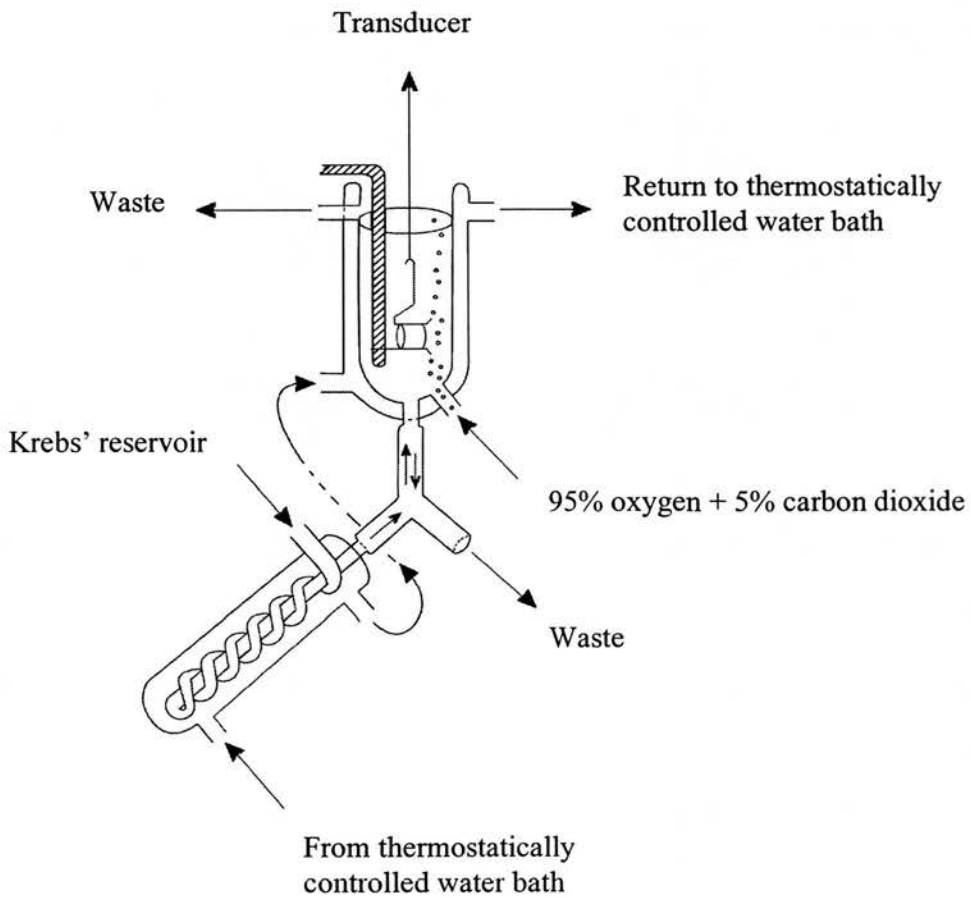

Figure 2.1: Experimental set-up for the measurement of tension of human hepatic artery rings without endothelium. This double jacketed tissue bath was used for all experiments, where recordings of isometric contractions were made. The volume of the bath is 10 ml.

Test preparations are usually washed by overflow, which means that the preparation is never without the physiological solution it needs. The two main purposes of washing are to provide fresh physiological solution and to wash out test compounds that have been added during the study. Since overflow washout is actually a dilution process, an excess volume of fresh solution must be added, i.e. about 5 times the volume of the inner bath. The preparation is usually washed twice. The volume of saline required to change the contents of a particular organ bath can be calculated by adding a dye to the full bath and measuring the volume of fresh water that has to be passed through before the contents are completely clear again. It is essential that the temperature of the washout solution does not vary by more than $\pm$ 0.5^0 C from the solution already in the inner bath. The reservoir of physiological saline used for washouts is maintained at a constant temperature with the help of thermostatic mechanisms and should also be constantly aerated with 95% O_2 +5% CO_2 passed through a large frit, or pumped in directly, in which case the reservoir should also be stirred (e.g. with a magnetic stirrer) in order to ensure homogeneity.

The inner organ bath chamber can have a volume of 50 mls. If only a small quantity of the test substance is available, however, a smaller vessel should be used, e.g. 5 or 10 mls. It is then particularly important to measure the dose precisely, and to add it to the bath in exactly the same way each time. A micropipette or a Hamilton syringe is sufficiently accurate to measure such small volumes. Although baths with a relatively large volume such as 50 mls require far more physiological solution and test substance, the possibility of error in measuring the dose is less, as the volumes involved are greater.

The lower end of the organ preparation can be attached to a glass hook fused to the floor of the inner bath, although it is quite difficult to loop the thread or wire clip around it successfully, because the preparation has to be kept relatively taut, so that it will not detach itself. The wire loop at the top end of the tissue is connected to the transducer to provide transmission, taking care not to overstretch the preparation. The organ bath must be deep enough for the organ and the wire clip to remain completely submerged in the physiological solution at all times.

Physiological salt solution

The current experimentation was done using KHS as the physiological solution to preserve recipient and donor hepatic arteries during transportation from the transplant theatre to the laboratory and during experimentation.

The constituents and preparation of the solution is shown in Table 2.1 below.

pH 7.4

Table 2.1:Constituents of KHS.

Constituent	Concentration (mM/l)
NaCl	118.5
KCl	4.7
$MgSO_4.7H_2O$	1.2
Na HCO_3	25
KH_2PO_4	1.2
Ca $Cl_2.6H_2O$	2.5
Glucose	11.6

Preparation of KHS

Working solutions of KHS were prepared from stock solutions 'A' and 'B' as required. The constituents of these solutions are shown in Table 2.2 (next page).

Table 2.2: Constituents of Solutions A and B.

Solution A		**Solution B**	
Constituent	Quantity	Constituent	Quantity
Deionised water	2 litres	Deionised water	2 litres
NaCl	276.8 gm	$NaHCO_3$	84 gm
KCl	14.0 gm	KH_2PO_4	6.4 gm
$MgSO_4.7H_2O$	11.6 gm		

Final KHS

This was prepared by adding together the following constituents.

Deionised water	1.8 litres
Solution A	100 ml
Solution B	100 ml
$CaCl_2.6H_2O$	1.1 gm
Glucose	4.2 gm

Preparation of other Stock solutions and calculation of volume to be added in the bath.

The following stock solutions were prepared using formula weights. Stock solutions of PE, L-NNA and D-NNA were prepared in deionized water and frozen in I ml. Aliquots. These were thawed as required and any residue was discarded at the end of experiment.

1) L-NNA:

Formula weight = 219.2

219.2 gm in 1 litre = 1 M

219.2 mg in 1 ml = 1 M

219.2 mg in 10 ml = 0.1 M

21.92 mg in 10 ml = 0.01 M (Stock Solution).

2) D-NNA:

Formula weight = 219.2

219.2 gm in 1 litre =1 M

219.2 mg in 1 ml = 1 M

219.2 mg in 10 ml = 0.1 M

21.92 mg in 10 ml = 0.01 M (Stock solution).

3) PE:

Formula weight = 203.7

203.7 mg in 1 ml = 1 M

203.7 mg in 10 ml =0.1 M

20.37 mg in 10 ml = 0.01 M (Stock Solution)

4) KCl:

Formula weight = 74.55

74.55 gm in 1 litre =1 M

7.455 gm in 100 ml =1 M

14.91 gm in 200 ml = 1 M

29.820 gm in 200 ml = 2 M

The volume of KCl to be added to the bath chamber containing the test preparation was calculated as follows:

If 2M KCl stock solution is to be added to an organ bath of 10 ml volume to give a concentration of 100 mM.

If C_1 = concentration of stock solution

V_1 = volume of stock solution added to the bath

C_2 = Final concentration of solution in the bath

V_2 = volume of the bath (=10 ml)

Apply the formula $C_1 \times V_1 = C_2 \times V_2$

Where $C_1 = 2M$

$V_1 = ?$

C2 = 100 mM=0.1 M

$V_2 = 10$ ml

$$V_1 = \frac{C_2 \times V_2}{C_1}$$

$$V_1 = \frac{0.1M \times 10\ ml}{2M} = 0.5\ ml = 500\ \mu\ litre$$

2.2.2 NOS inhibition and its impact on ∝-adrenoceptor mediated contraction *invitro*

2.2.2.1 Background:

Patients with cirrhosis show impaired responsiveness to vasoconstrictors, which is maintained *invitro* (Hadoke PWF and Hayes PC, 1997). The reason for this is not clear, although various mechanisms have been proposed. These include the effect of vasodilatory agents (Loyke et al, 1986), receptor down-regulation (Gerbes et al, 1985) or a post-receptor defect (Murray and Paller, 1985), (for details see section 2.1-2.113). Generation of NO by iNOS in vascular smooth muscle cells may contribute to impaired contractile function in arteries from patients with cirrhosis. This is controversial, as studies of isometric contraction in humans have produced conflicting results (Smith et al, 1993; Schepke et al, 1997).

2.2.2.2 Aim:

The study aimed to determine whether the release of NO from vascular smooth muscle cells modulates α-adrenoceptor-mediated contraction of endothelium denuded hepatic arteries isolated from patients with cirrhosis.

2.2.2.3 Materials and Methods:

Vessel Acquisition and Preparation

For the study of vascular reactivity in splanchnic vessels isolated from patients with cirrhosis, hepatic arteries were obtained from (non-cirrhotic) liver donors (n=8) and (cirrhotic) recipients (n=9) at the time of OLT at the SLTU at the Royal Infirmary of Edinburgh, Scotland. The cause of death of donors and aetiology of cirrhosis in recipients are given in Table 2.3.

During retrieval of the healthy liver from an organ donor, the surgical team routinely collects the entire hepatic artery and the coeliac axis. These are stored in a preservative, UWS (UWS; NPBI International BV, Emmer-Compascum, The Netherlands; and Du-Pont Pharmaceuticals Ltd., Herts, UK.) at 4^0 C with the donor liver. At the end of OLT, after anastomoses of the graft, the residual hepatic artery samples are usually discarded and so become available for functional analysis. As the (cirrhotic) liver removed from the recipient is fixed in normal saline for histological analysis, recipient hepatic arteries were dissected directly from the explanted recipient livers, prior to fixing immediately after it had been removed from the patient.

Donor and recipient hepatic arteries obtained from the transplant theatre were placed immediately in fresh, ice-cold, oxygenated KHS and transported to the laboratory for functional analysis. This provided donor hepatic arteries which had been stored in UWS for up to 16 hours and recipient hepatic arteries, obtained immediately following removal from the patients.

Table 2.3: Causes of death in liver donors and aetiology of cirrhosis in recipients.

Donor	No.	Recipient	No.
Sub-arachnoid haemorrhage	3	Primary Biliary Cirrhosis	6
Intracerebral haemorrhage	3	Alcoholic Liver Disease	1
Head injury	1	Primary Sclerosing Cholangitis	1
Road Traffic Accident	1	Autoimmune Hepatitis	1
Total	8	Total	9

Ethical approval for use of human tissues was obtained from the Lothian Research Ethical Committee and the arrangements were approved by the United Kingdom Transplant Support Services Authority.

These arteries were carefully cleaned of adherent connective tissue and fat, and if necessary, were stored in the refrigerator at 4^0 C in freshly prepared oxygenated KHS solution. Whenever possible, experiments were performed immediately after the retrieval of the arteries. Such vessels are generally devoid of endothelium and removal of any residual endothelium was ensured by rubbing the luminal surface of each vessel with a blunt probe, while the rings were kept completely immersed in KHS on a petri-dish.

Experimental Protocol

Two rings (2 mm in length) were prepared from each artery and mounted in parallel on two intraluminal stainless steel wire hooks in 10 ml. organ bath chambers (described in section 2.2.1) containing KHS (composition as shown above) at 37^0C continuously perfused with 95%O_2 and 5%CO_2. The lower hook was fixed to the base of the organ bath, whilst the upper hook was attached to a force displacement transducer (Grass FT-03) connected to a computerized data acquisition system (Dr H Brash, Department of Medicine, Royal Infirmary of Edinburgh) for the measurement of isometric force.

The arterial rings were then gradually stretched to their optimum resting force [previously shown to be 4g (Hadoke et al., 1998)] and allowed to equilibrate for 45-

60 min. During equilibration, the tension was gradually adjusted and the contents of the organ baths replaced at frequent intervals. After equilibration, the viability and reproducibility of contraction of each ring was tested by stimulating with a single concentration of 100mM KCl. Rings were washed with KHS once the contraction was stabilized. The procedure was repeated 3-4 times until reproducible, sustained contractions were obtained.

One ring from each artery was incubated for 40min with the NOS inhibitor L-NNA; (10^{-4}M), whilst the other was incubated with its inactive isomer D-NNA; (10^{-4}M). Cumulative concentration response curves were then constructed for PE; (10^{-9}-$3x10^{-4}$M) and KCl; (2.5-120 mM).

L-NNA, D-NNA and PE were obtained from Sigma, Poole, Dorset, UK.

Sample traces of blood vessel contractility are shown in Appendix 1-1V.

Statistical analysis:

Results are expressed as mean ± SEM; n represents the number of subjects. Maximal contractions are given in grams and as a percentage of maximum contraction to KCl to control for variation in the size of the arterial rings.Sensitivity (pD_2) values for agonists were determined for individual concentration-response curves using a curve-fitting programme (Fig P., Biosoft, Cambridge, U.K). M.C. contraction and sensitivity (pD_2) values were compared using Student's unpaired t-test and values were considered significant when $p<0.05$.

2.2.2.4 Results:

For each artery, mean values were calculated from the results of 2 rings, where appropriate. The maximum contractile responses of the receptor-dependent agonist (PE) are given as gms. and as a percentage of the maximum contraction to KCl (to minimize any discrepancy in the sizes of the arterial rings (Lew and Angus, 1992)). Contractile responses to receptor independent agonist (KCl) are expressed in gms. (Table 2.4).

Denuded hepatic arteries obtained from non-cirrhotic donors and cirrhotic recipients contracted in a cumulative concentration-dependent way, when exposed to receptor-dependent agonist (PE) and receptor-independent agonist (KCl).

The maximum contractile response to PE in RHA (M.C. 9.33±1.09 g, n=9) was similar to that of DHA (M.C. 10.46±1.71 g, n=8, p=0.58). Similarly, the contractile response of RHA and DHA was not dissimilar when expressed as a percentage of maximum contractile response to KCl (M.C.122.44±11.16% and 155.51±17.26%; p=0.20; RHA and DHA respectively). The pD_2 values for this agonist between RHA (5.26±0.22) and DHA (5.67±0.31) were also similar (p=0.40) (Table 2.4), despite a suggestion that PE caused a smaller, less sensitive, response in arteries from (cirrhotic) recipients, compared with (non-cirrhotic) donors (Figure 2.2).

The denuded hepatic arterial rings obtained from the (cirrhotic) recipients and (non-cirrhotic) donors, contracted in a cumulative concentration-dependent way, when

exposed to PE and KCl, whether incubated with L-NNA or D-NNA. Incubation of these denuded arterial rings with NOS inhibitor L-NNA had no effect on basal resting tone set up during equilibration, in both DHA and RHA. Furthermore, incubation with L-NNA did not alter the response of either DHA (Figure 2.3) or RHA (Figure 2.4) to PE (Table 2.5). Cumulative concentration response curve produced in response to KCl in DHA and RHA is shown in Figure 2.5. This curve shows that the receptor independent contraction to KCl is maximal at 100-120 mM KCl concentration.

Table: 2.4: PE response comparing DHA and RHA.

	RHA (n=9)	DHA (n=8)	*p* value
M.C.(g)	9.33 ± 1.09	10.46 ± 1.71	0.58
M.C. (%KCI)	122.44 ± 11.16	155.51 ± 17.26	0.20
PD_2	5.26 ± 0.22	5.67 ± 0.31	0.40
M.C. to KCI (g)	7.81 ± 1.10	7.2 ± 1.32	0.72

Results are expressed as mean ± s.e.m. in (n) arteries.

M.C. = Maximum Contraction

pD_2 = -log EC 50, where EC 50 is the concentration of the test substance in the bath which produces half maximal contraction.

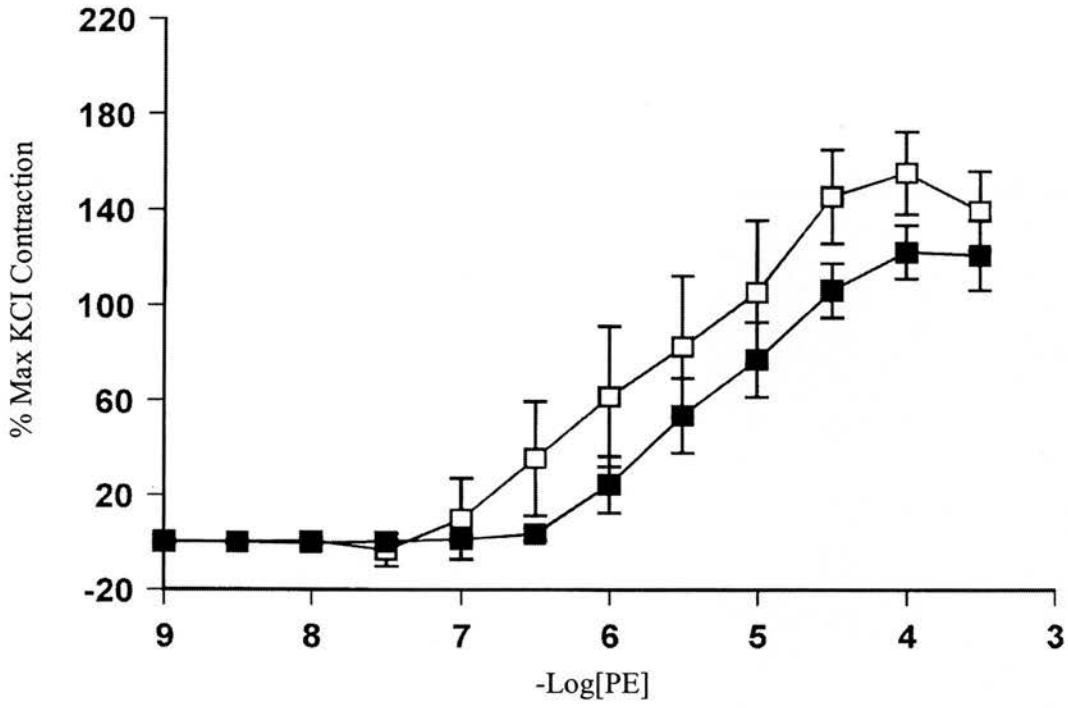

Figure 2.2: Cumulative concentration-response curves produced in response to PE by DHA (□) and RHA (ν). Responses are given as mean ± s.e.m. and expressed as a percentage of the maximum contraction induced by KCl. For n values see Table 2.4.

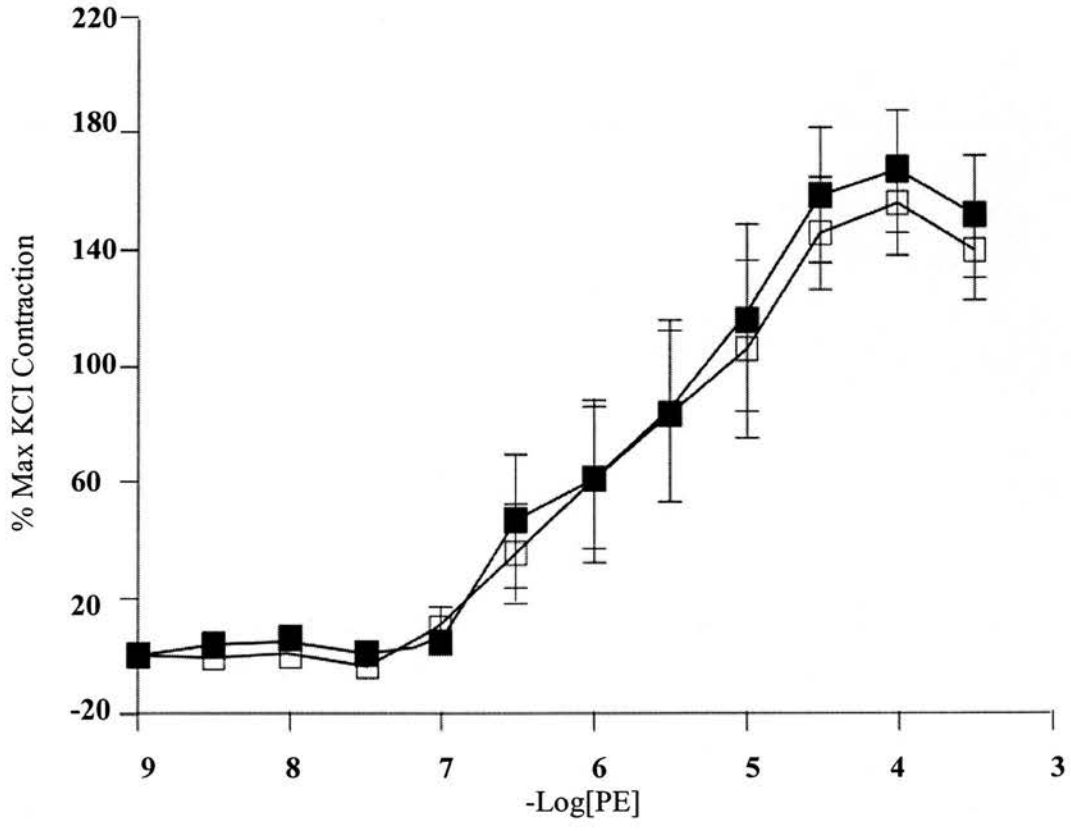

Figure 2.3: Cumulative concentration-response curves produced in response to PE by DHA after incubation with D-NNA (□) and L-NNA (■). Responses are given as mean ± s.e.m. and expressed as a percentage of the maximum contraction induced by KCl. For n values see Table 2.4.

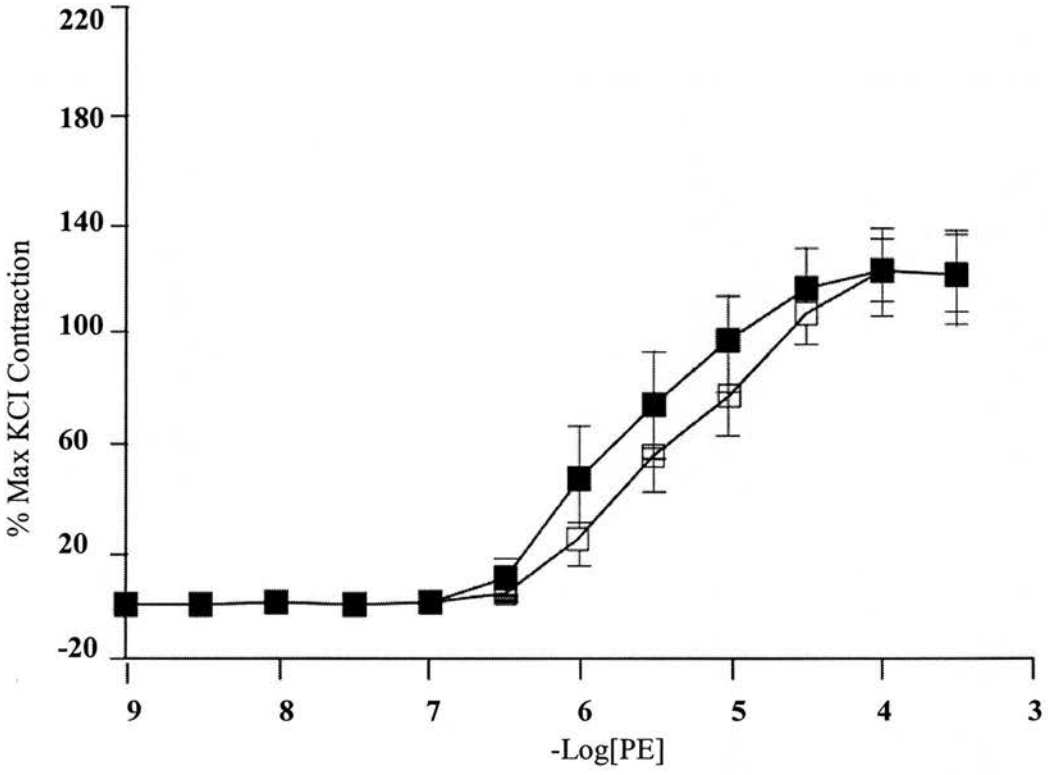

Figure 2.4: Cumulative concentration-response curves produced in response to PE by RHA after incubation with D-NNA (□) and L-NNA (v). Responses are given as mean ± s.e.m. and expressed as a percentage of the maximum contraction induced by KCI. For n values see Table 2.4.

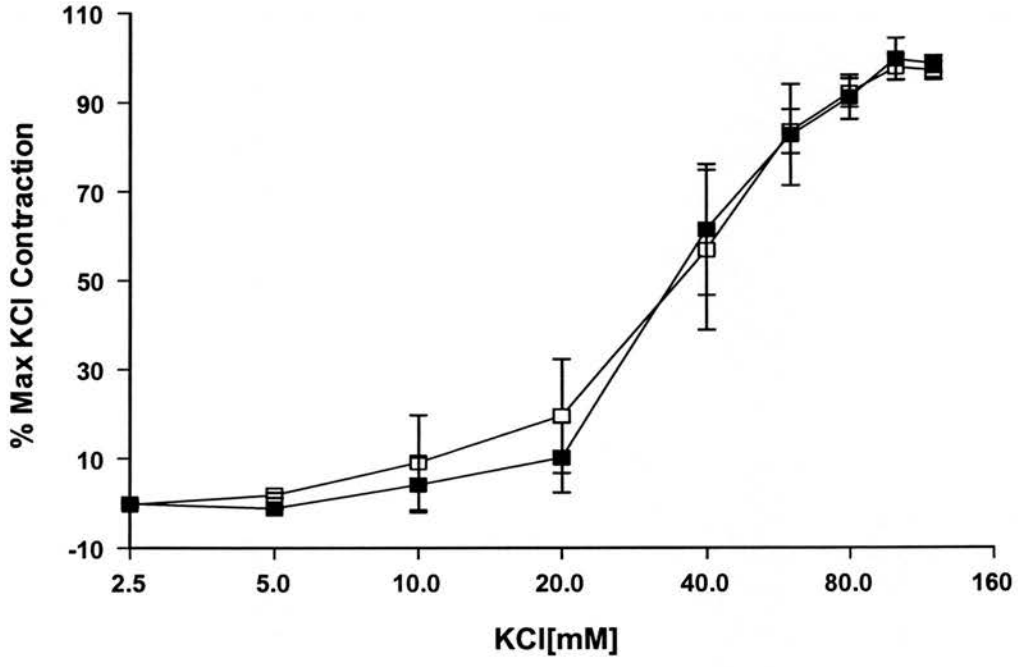

Figure 2.5: Cumulative concentration response curves produced in response to KCl by DHA (□) and RHA (■). Responses are given as mean ± s.e.m. and expressed as a percentage of the maximum contraction induced by KCl. For n values see Table 2.4.

Table: 2.5: PE response in DHA and RHA after incubation with D-NNA and L-NNA.

	RHA (n=9)			DHA (n=8)		
	D-NNA	**L-NNA**	***p value***	**D-NNA**	**L-NNA**	***p value***
M.C.(g)	9.33 ± 1.09	11.46 ± 1.24	0.21	10.46 ± 1.71	11.25 ± 1.83	0.75
M.C. (%KCI)	122.44 ± 11.6	121.46 ± 15.96	0.94	155.51 ± 17.26	166.99 ± 21.21	0.66
pD_2	5.26 ± 0.22	5.62 ± 0.2	0.25	5.67 ± 0.31	5.67 ± 0.27	0.86

Results are expressed as mean ± s.e.m. in (n) arteries

M.C. = Maximum Contraction

pD_2 = -log EC 50

2.2.2.5 Discussion:

The present investigation has studied vascular responsiveness of endothelium denuded hepatic arteries in portal hypertension due to cirrhosis of the liver in human subjects. The role of iNOS in modulating vascular contractility has been specifically studied.

It has been proposed that the impaired pressor response to α-adrenoceptor agonists in patients with advanced cirrhosis is due to inability of vessels to respond to constrictor agonists. The persistence of impaired contraction *exvivo* in arteries isolated from animal models suggests that this could be due to an intrinsic defect in the cells of the vessel wall rather than being secondary to the action of a circulating vasodilator substance (reviewed in Hadoke and Hayes, 1997). The current investigation found no difference in the α-adrenoceptor mediated, nor receptor independent contractile response between endothelium denuded hepatic arteries obtained from cirrhotic and normal humans (Table 2.4). This indicates that the impaired excitation-contraction coupling in, or the release of vasodilators from the vascular smooth muscle cells of hepatic arteries, are not responsible for the impaired pressor response associated with this condition and that circulating vasodilators or endothelium derived factors are probably active *invivo* in modulating vascular responses to physiological stimuli.

Hepatic arteries were used in this study, as they belong to a vascular territory (splanchnic), which is known to be dilated in cirrhosis (Iwao et al, 1997). They contracted in a stable reproducible manner when exposed to PE and KCl *invitro*.

Although, hepatic arteries with an intact endothelium have been obtained from hepatectomy specimens (Jeng et al, 1996), loss of endothelium is a consistent finding in all investigations using arteries from the transplant theatre (Smith et al 1993; Heller et al, 1996; Schepke et al, 1997). It was also anticipated that preservation in UWS would not alter the contractile function of donor hepatic arteries. It has been shown already that function of the rat mesenteric arteries is unaltered after 3 days in a physiological salt solution (McIntyre et al, 1998) and UWS has been shown to preserve the contractile function of human hepatic arteries (Jeng et al, 1996).

A defect in receptor-mediated contraction may occur as a result of either receptor down regulation or altered post-receptor signal transduction. In the current study, constriction in response to the selective α_1–adrenoceptor agonist PE was not altered in hepatic conduit arteries from transplant recipients. As the α_1–adrenoceptor subtype has been shown to mediate contraction in the hepatic artery (Vagianos et al, 1990), the results of the present investigation suggest this receptor is not down regulated in hepatic arteries of patients with cirrhosis. Normal receptor function in arteries from animals with cirrhosis or portal hypertension (Liao et al, 1994) suggests that the impaired contraction was the result of a post receptor defect in signal transduction mechanisms. Such a defect in the hepatic artery was not indicated in the present study as neither receptor mediated (PE) nor receptor independent (KCl) contractions were impaired.

Furthermore, the inhibition of iNOS by incubation with L-NNA made no difference to the contractile response in the recipient (cirrhotic) and donor (normal) hepatic arteries, suggesting that the release of NO by the vascular smooth muscle cells does not occur in human hepatic arteries as this would have resulted in reduced responses to both receptor-dependent and receptor independent vasoconstrictors (Jeng et al, 1996). This is consistent with other results presented recently where hepatic arteries isolated from the transplant theatre were used (Schepke et al, 1997). The loss of endothelium from the vessels in the current study prevented investigation of the role of NO released by eNOS.

These results indicate that future investigations should attempt to clarify the role of the endothelium in mediating hyporesponsiveness to vasoconstrictors in patients with cirrhosis.

2.2.2.6 Conclusion:

These results indicate that the release of NO from the smooth muscle cells of the hepatic artery does not modulate α-adrenoceptor-mediated contraction in patients with cirrhosis.

SECTION 3

SPLENOMEGALY IN PORTAL HYPERTENSION DUE TO HEPATIC CIRRHOSIS

3.1 INTRODUCTION

Cirrhosis of the liver is a chronic disorder which leads to portal hypertension and hepatic failure in the end stage of the disease. Portal hypertension in hepatic cirrhosis results mainly from increased resistance to portal blood flow and leads to the development of porto-systemic collaterals, which partly decompress the portal circulation. At the same time, the portal venous inflow through the splanchnic circulation increases and thus maintains the elevated portal pressure and also compensates, to some extent, for the loss of portal blood flow through collaterals. The development of a collateral circulation is partly responsible for hepatic encephalopathy and leads to gastro-intestinal bleeding from GOV and portal gastropathy.

3.1.1 Splenomegaly:

Portal haemodynamic changes in cirrhosis lead to the development of splenomegaly. This occurs earlier in portal hypertension than the development of GOV and care is needed in diagnosing portal hypertension without the demonstration of splenomegaly (Hayes PC, 1997).

The clinical diagnosis of splenomegaly itself is difficult, because the organ has to enlarge considerably before becoming palpable and therefore more sensitive methods to measure spleen size are required. A number of techniques have been used to measure spleen size and these include abdominal radiography (Martin, 1937), ultrasonography (Koga and Morikawa, 1975; Niederan et al, 1983), CT (Heymsfield et al, 1979), MRI (Williams et al, 1985) and radionuclide scanning

(Rollo et al, 1970; Mut et al, 1988; Strause et al, 1984). The accuracy and comparability of these techniques is not well established as relatively few studies have investigated their comparability and only limited numbers of patients have been studied (Meek et al, 1984).

3.1.1.1 Clinical examination of the Spleen:

Clinical examination of the spleen, although seemingly a simple bedside diagnostic tool, requires careful technique and the recognition of its limitations. Palpability of the spleen depends upon a number of factors including size of the organ, its position, laxity of the ligaments which hold it in place, the thickness and tension of the abdominal muscles, obesity and the presence of ascites. In practice, a palpable spleen is almost always enlarged, but an impalpable spleen is not necessarily of normal size. The increasing precision of radiological techniques however, does not diminish the value of bedside methods of detecting splenomegaly.

Clinical examination is best carried out from the right side of the patient. Palpation is begun in the right iliac fossa to avoid missing a grossly enlarged spleen and the right hand is advanced towards the left hypochondrium. Minimal degrees of palpable enlargement are detectable by placing the hand in the left loin to lift the lower aspect of the rib cage forward and then attempting to feel for the lower pole or the medial border of the minimally enlarged spleen. This manouvre displaces the spleen forward and aids it to become palpable. Clinical palpation of spleen is a skill learnt with great practice. Percussion over the lower ribs may prove to be a useful adjunct. The long axis of the spleen lies along the 10^{th} rib and with enlargement,

extends medially and downwards (Figure 3.1). Splenic dullness to percussion, in health rarely extends further forwards than the mid-axillary line. A change in the percussion note in the anterior axillary line on inspiration with the patient lying supine may indicate early enlargement (Castell, 1967).

3.1.1.2 Structure of the spleen

Gross anatomy and size

The spleen is an important lymphoid organ. Its size is very variable, ranging from 35 gms. in elderly females to 280 gms. in young adults (Krumbhaar and Lippincott, 1939; Deland, 1970; Myers and Segal, 1974). At birth, the spleen is about 4.5 cm. in length, 2.5 cm. wide and 1.5 cm. thick, and the mean weight is 11 gms. By the age of one year the weight is 15-25 gms; by 5 years it is 40-70 gms. and by 10 years it is 80-100 gms. (Lewis, 1987). It reaches its maximum weight at around puberty, when it has a mean weight of about 150-180 gms. (Hanley and Ludlam, 1997). It tends to become lighter thereafter. It is slightly heavier in the adult male than in the female. The size of the spleen as assessed by plain X-Rays of the abdomen, ultrasonography, CT scanning, MRI and scintigraphic radionuclide studies has revealed, that the normal adult spleen is 8-11 cms. long, 4.5-6.0 cms. wide and has a volume of less than 250 mls. Spleens larger than this are usually palpable.

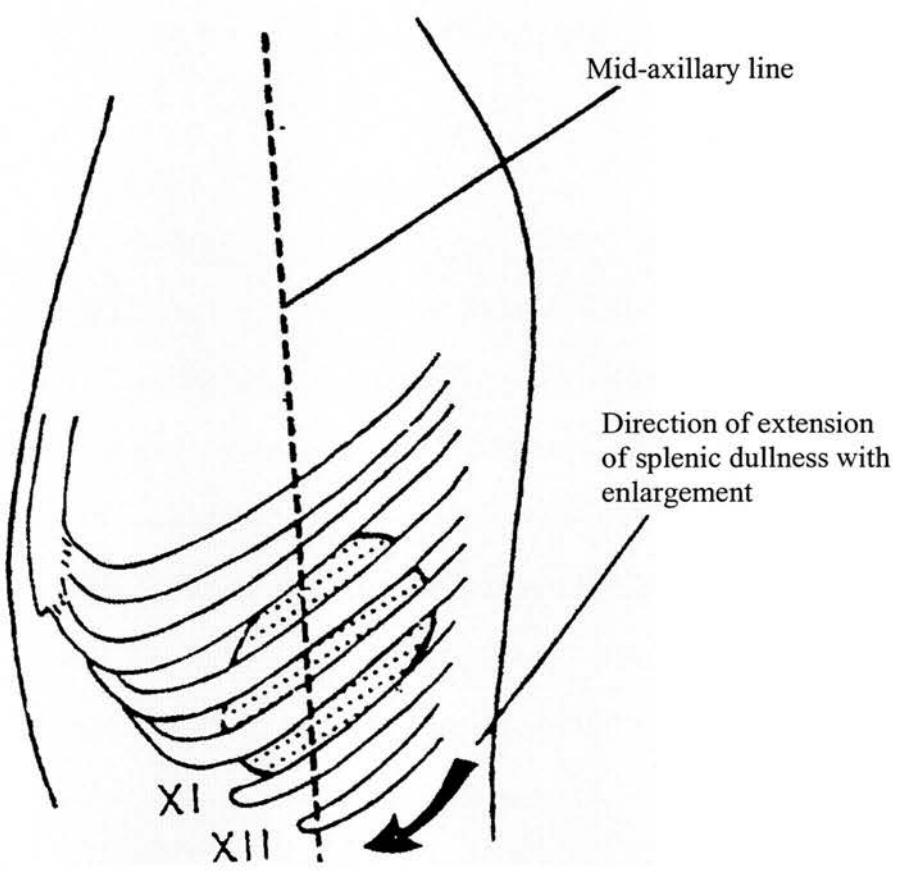

Figure 3.1: An early physical sign of splenic enlargement; extension of splenic dullness anterior to the mid-axillary line along the line of the 10^{th} rib.

The size of spleen however, cannot be the only factor to determine palpability, as the organ can be palpable in normal young adults without being enlarged (Berris, 1966; Ebaugh and McIntyre, 1979) and a grossly enlarged spleen may not be palpable. Fischer (1970) observed that 50% of moderately enlarged spleens weighing 600 to 750 gms. and 20% of greatly enlarged spleens weighing 900 to 1600 gms. could not be palpated. The presence of ascites makes clinical detection even more difficult (Fischer, 1970). This highlights the importance of radiological aids like abdominal ultrasonography, CT scanning, MRI and scintigraphy in verifying and supplementing the findings of clinical examination.

Histology

The spleen has a complicated structure. It consists of a connective tissue framework, vascular channels, lymphatic tissue, lymph drainage channels, and cellular components of the haematopoeitic and R-E systems. Histologically, there are two main components: (a) the white pulp; and (b) the red pulp. The white pulp consists mainly of peri-arteriolar lymphoid sheaths and the adjoining follicles (Malpighian bodies) which contain a germinal centre and are structurally similar to lymphoid follicles. The red pulp consists of sinuses, lined by macrophages and pulp cords. The outer capsule of connective tissue framework consists of collagen tissue covered by serous endothelium. From this, come off a large number of trabeculae which extend into the pulp, carrying blood vessels and autonomic nerves. The spleen is a very vascular organ and derives its arterial blood supply from the splenic artery which is a branch of the coeliac axis and thence through the trabecular arteries, into the central arteries which are sited in the white pulp. These latter vessels run into a

central axis of the periartiolar lymphatic sheaths; and give off many arterioles and capillaries, some of which terminate in the white pulp whilst others go into the red pulp. There they either connect directly with the sinuses and thence, via the collecting vein, to the trabecular veins or they first pass into the cord spaces before passing into the sinuses (Figure 3.2).

Thus, as the blood flows through the spleen it comes into contact with the reticular fibres and with the reticulo-endothelial macrophages which lie in the interstices of the reticular mesh. The splenic vein drains the blood into the portal venous circulation.

Blood flow in the spleen

The spleen seems to have two blood flow systems (closed and open), whereby a rapid and slow circulation occurs through the organ (Fig. 3.2). The slower component is the usual pathway under normal circumstances, and several passages of the whole blood volume through it occur during a 24 hour period. In splenomegaly, the blood flow through the spleen can reach 20 percent of the blood volume per minute. At the same time, increased pooling of blood also occurs in the enlarged organ. (Harris et al, 1958; Christensen, 1973).

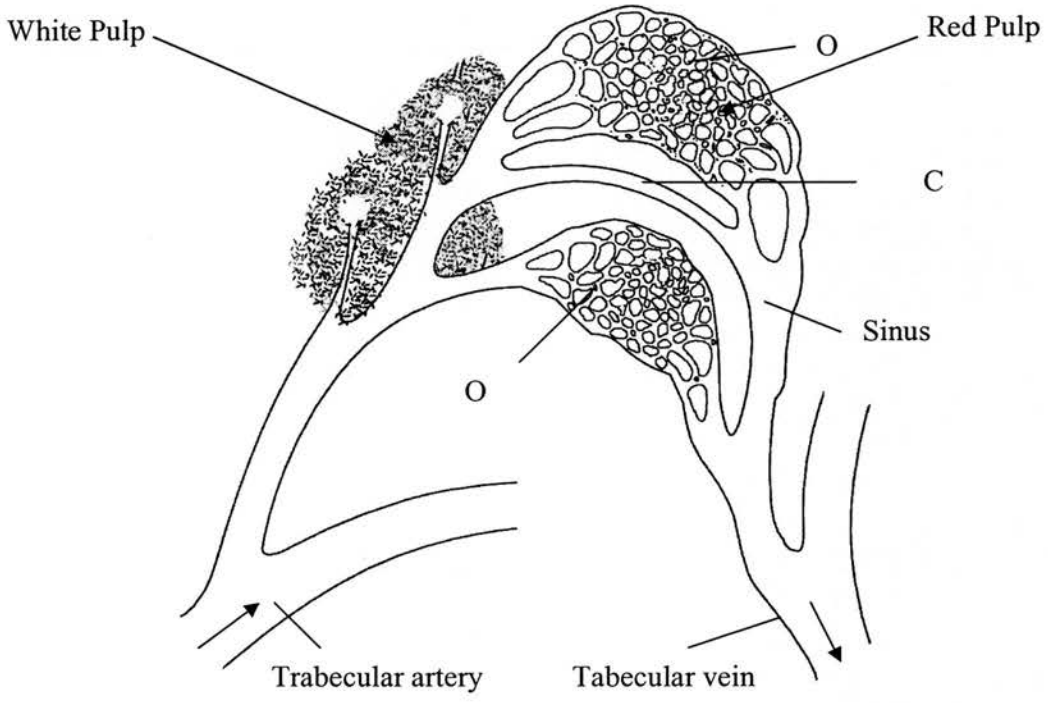

Figure 3.2: Diagramatic illustration of the circulation of the spleen. The blood passes either directly into a sinus (C, closed system) or first into the cord spaces in the red pulp (O, open system).

3.1.1.3 Function:

Cell sequestration, phagocytosis and pooling in the spleen

The spleen has a remarkable ability to aid in the maturation of red blood cells for recirculation and also to remove from the circulation effete or damaged cells as well as foreign matter. This occurs via three different mechanisms: *sequestration* which is a temporary (reversible) process whereby cells are held in the spleen before returning to the circulation; *phagocytosis* which represents the irreversible uptake of non-viable cells by macrophages or destruction of viable cells which have been damaged in some way; *pooling* which is the presence in the spleen of an increased amount of blood. In contrast to sequestration, pooled cells are in continuous exchange with the circulation.

Hypersplenism

Hypersplenism is a clinical syndrome of varied aetiology. It is characterized by:

a) Splenomegaly, although this may be only moderate.
b) A reduction in the number of one or more of the cellular components leading to thrombocytopenia, neutropenia, anaemia and even pancytopenia.
c) Normal or increased production of the precursor cells in the marrow.
d) Premature release of cells into the peripheral blood, resulting in a mild reticulocytosis with nucleated red cells and occasional immature granulocytes.
e) Decreased red cell survival.
f) Decreased platelet survival.
g) Hypervolaemia, if splenomegaly is marked.

The central role of the spleen in producing hypersplenism is confirmed by the finding of reversal of the abnormality in haematological counts following splenectomy.

3.1.1.4 Pathogenesis of splenomegaly in portal hypertension:

The pathogenesis of splenomegaly in portal hypertension due to hepatic cirrhosis is not clear. It is generally believed that passive vascular congestion due to back pressure on the splenic circulation from the hypertensive portal vein is responsible for enlargement of the spleen. However, this may not be the only factor determining spleen size, as splenomegaly does not reverse totally with decompressive portosystemic shunt surgery or with the insertion of TIPSS (Jalan et al, 1996b). Further, there is evidence of splanchnic hyperaemia and increase in splenic arterial blood flow with the development and progression of portal hypertension in hepatic cirrhosis. Size of the splenic artery and blood flow, as assessed by ultrasonography and duplex doppler scanning, correlates with the size of the spleen. However, this could be the result of splenomegaly rather than the cause of it. There is also evidence that lymphoid tissue hyperplasia could be a factor in producing splenomegaly in hepatic cirrhosis. This is supported by autopsy studies in humans (McMichael, 1934) as well as experimental data from animals with portal hypertension where it has been shown that splenic tissue implants grow and replicate lymphoid cells actively after implantation into the subcutaneous tissues implying the importance of factors other than portal congestion in the production of splenomegaly in liver disease (Dumont et al, 1974). In summary, at least three factors seem to

participate in the production of splenomegaly, i.e., portal hypertension, increase in splanchnic blood flow and lymphoid hyperplasia.

3.1.2 Splanchnic blood flow in portal hypertension

Normally, the portal venous system conveys about 1.0-1.5 litres of blood/min to the liver. The normal portal venous pressure is in the range 4-6 mm Hg. In portal hypertension, there is a reduction of hepatopetal flow to a variable degree. This is most marked in the extra-hepatic variety of portal hypertension. Reduction of portal vein blood flow in patients suffering from cirrhosis has been recorded as 450 ± 350 mls./min (range 0-1690 mls./min) (Moreno et al, 1975).

Although, there is a general agreement, that portal blood flow is reduced in portal hypertension, there is some disagreement about the frequency of retrograde blood flow (centrifugal to the liver) in the portal vein. Radiological studies of the portal circulation have certain limitations but the direct introduction of contrast material into the portal vein shows that retrograde flow can occur, usually in patients with large collateral vessels (Takayasu et al, 1982; Rector et al, 1988).

3.1.2.1 Portal-systemic shunts and collateral vessels

Almost all of the portal venous blood normally flows towards the liver, but in portal hypertension a variable amount is shunted into the systemic circulation through collateral vessels or by intra-hepatic shunts, which bypass the sinusoids and join the hepatic veins directly. The proportion of the portal blood shunted varies and may reach 100 percent (Lebrec et al, 1976; Okuda et al, 1977). Collateral vessels are an important sign of raised portal pressure. They can occur at different sites. The most

important ones, clinically are in the stomach and oesophagus, where they may bleed (Figure 3.3). They may also occur in the rectum and anus, in the anterior abdominal wall, around the umbilicus, behind the colon and duodenum and over the bare area of the liver. The extent of shunting increases with time and presumably, therefore with the progression of liver disease. Shunting is especially marked in patients who suffer from gastro-oesophageal variceal bleeds (Groszmann et al, 1972; Lebrec et al, 1976).

The major risk factor for variceal haemorrhage seems to be the size of oesophageal varices and it has been suggested that blood flow through the varices is a more important risk factor than portal pressure (Sauerbruch et al, 1988). The direct measurement of blood flow through oesophageal varices remains difficult to achieve, but it has been proposed that blood flow in the azygos vein into which the varices drain, reflects blood flow in the varices. Blood flow in the azygos vein in individuals without portal hypertension is about 175 mls./min. (Bosch et al, 1985). Factors such as propranolol, which cause a reduction in the azygos blood flow (Lebrec et al, 1981; Bosch et al, 1984) are believed to also reduce variceal blood flow which may be important in reducing the risk of variceal haemorrhage. Thus, blood flow through the azygos vein reflects the degree of shunting through gastro-oesophageal collaterals, and the azygos blood flow in patients who have bled from varices reaches about 600 mls./min. Until direct measurement of variceal blood flow becomes routinely possible, recording azygos blood flow remains an important tool for investigation of portal haemodynamics.

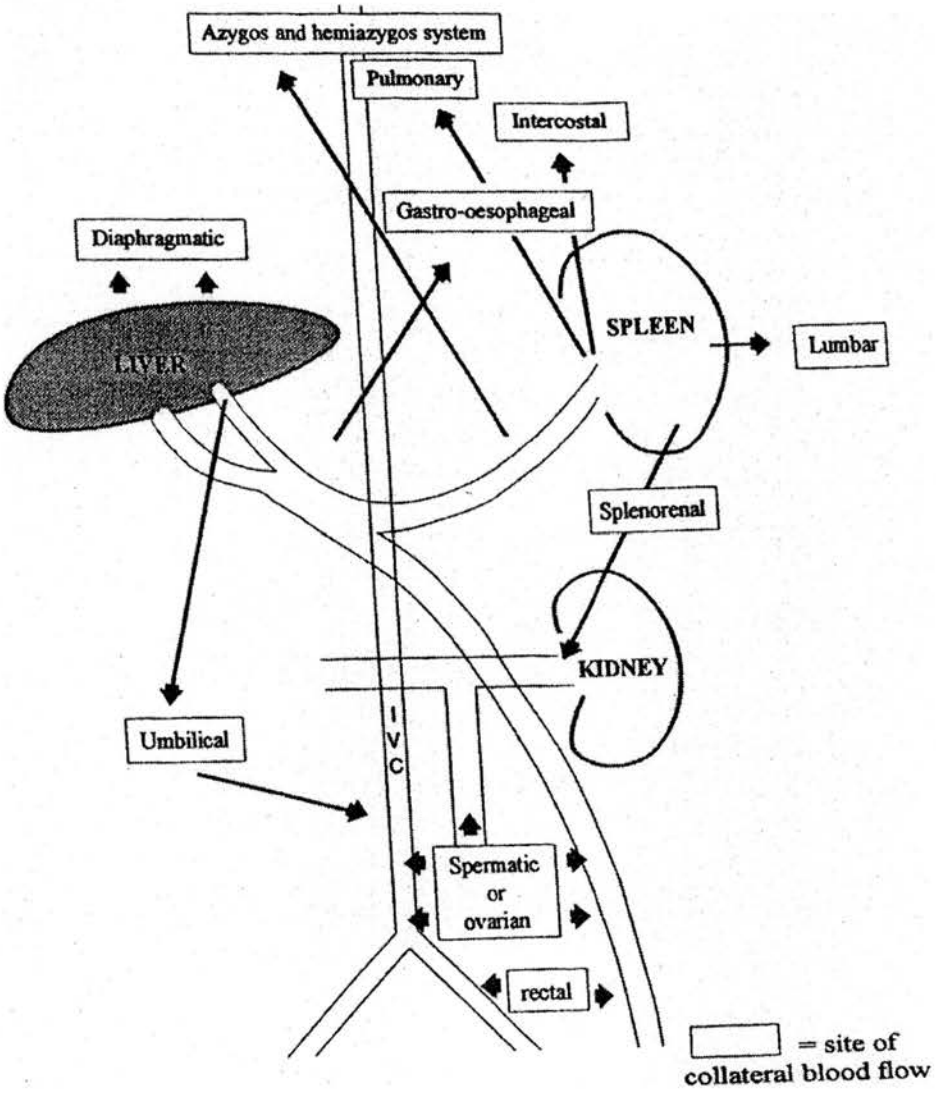

Figure 3.3: When the portal circulation is obstructed, collateral circulation develops to return the portal blood into the systemic veins.

3.1.3 Evaluation of portal hypertension and liver function in hepatic cirrhosis.

The haemodynamic alterations in the portal circulation due to hepatic cirrhosis are complex. Various methods have been used to visualize the portal system. Angiographic procedures can visualize the portal system very well (Burcharth, 1979) but their invasiveness and cost precludes their use for screening or follow up. CT scanning (Ishikawa et al 1980; Waller et al, 1984) and MRI (Ohtomo et al, 1986) can delineate intra-abdominal vessels well, but assessment of direction and velocity of flow are difficult. On the other hand, ultrasound is a less expensive, non-invasive, readily available and relatively accurate method of investigation (Dach et al, 1981).

3.1.3.1 Abdominal ultrasonography and doppler ultrasound

Ultrasound can visualize the portal system accurately (Dach et al, 1981; Dokmeci et al, 1981; Juttner et al, 1982; Kane et al, 1982; Hill et al, 1985; Subramanyam et al, 1983) and doppler adds reproducible information about the presence, direction and velocity of portal blood flow (Patriquin et al, 1987). Therefore, doppler ultrasound has advantages over other imaging modalities to make it a good procedure for diagnosis and follow up of portal hypertension.

Taylor (1975) noted that a large portal vein on ultrasound was a sign of portal hypertension. Since then a number of studies have been undertaken to differentiate normal from portal hypertensive patients on the basis of portal vein diameter measurements (Bolondi et al, 1984; Zoli et al, 1985; Kurol and Forsberg, 1986). Measurements should be performed in the fasting state and in the supine position

because the splanchnic vein diameters increase significantly (Bellamy et al, 1984; Rahim et al, 1985) after a meal and in the left lateral decubitus position.

Zoli (1985) examined 100 cirrhotics and 100 pair-matched controls and found that the sum of the expiratory diameter of the superior mesenteric vein and the splenic vein in excess of 9.2 mm/m^2 body surface area was a reliable sign of portal hypertension (sensitivity 91 percent and negative predictive value 92 percent) (Zoli et al, 1985). However, in an angiographic study on 64 cirrhotic patients and 33 controls, Lafortune (1984) found no correlation between elevated portal pressure and splenic vein diameter. In view of these discordant reports, the value of venous diameter measurements as a discriminatory test for portal hypertension is still controversial.

Another approach is to look at respiratory variations in venous diameter. In healthy individuals, the diameter of the superior mesenteric vein and splenic vein increase markedly during inspiration (Bolondi et al, 1984). This can be explained by mechanical compression of the liver during inspiration, leading to a decrease in hepatic outflow while splanchnic inflow continues, the net effect being distension of the splanchnic veins (Rabinovici and Navot 1980). These respiratory changes are significantly less or even absent in portal hypertensive patients.

The presence of collaterals is another highly specific sign of portal hypertension. Although large spontaneous shunts are easily detected by sonography, small collaterals can be difficult to resolve. This may be why the reported sensitivity for

demonstrating collaterals ranges from 12-85 percent (Dokmeci et al, 1981; Bolondi et al, 1984). A coronary vein diameter in excess of 5 mm is a further sign of portal hypertension, with a reported sensitivity of 64 percent (Subramanyam et al, 1983). A coronary vein more than 7 mm indicates severe portal hypertension with a danger of variceal bleeding (Lafortune et al, 1984).

Studies to estimate the volume of blood flow in portal hypertension using doppler ultrasound have been reported (Moriyasu et al, 1984; Ohnishi et al, 1985; Moriyasu et al, 1986; Sato et al, 1987). The principle of estimating volume flow by doppler insonation technique is simple: measure the average velocity of blood flowing in the portal vein using the doppler method, multiply by the cross-sectional area of the vessel, and thus obtain the flow volume. The ultrasonic duplex scanner combines pulsed doppler with real-time imaging, thus allowing both measurements to be made.

Although mean velocity and cross-sectional area measurements are subject to practical and theoretical problems, the results of these studies are more or less consistent. There is a significant decrease in mean portal blood flow velocity in portal hypertension (Moriyasu et al, 1984; Ohnishi et al, 1985), but the total portal flow volume often does not change and may even increase (Gill, 1985) due to a significant increase in cross-sectional area. This observation can be quantified in a *congestive index,* which is the cross-sectional area divided by the mean velocity. This is significantly higher in patients with portal hypertension compared to normal patients (Moriyasu et al, 1986).

3.1.3.2 WHVP and PPG.

The normal portal pressure ranges from 4-6 mm. Hg. and the pressure gradient between portal and hepatic veins ranges from 1-4 mm. Hg. Pressure values above these limits define the presence of portal hypertension. The formation of oesophageal varices requires a pressure gradient above 10 mm. Hg., and it has been proposed that the development of ascites requires a gradient above 8 mm. Hg (Bosch et al, 1986).

The measurement of portal pressure gives useful information in several clinical situations. The finding of a high portal pressure will confirm the presence of portal hypertension (Reynolds et al, 1970). Furthermore, it may be useful in the follow-up of patients after TIPSS (Dick et al, 1995), and to measure the response to drug therapy for the prevention of variceal haemorrhage (Bosch et al, 1993). It can also offer useful information about the cause of portal hypertension, because a normal WHVP is strongly suggestive of presinusoidal hypertension (Mc Cormick and Burroughs, 1990).

Portal pressure is well correlated with the severity of cirrhosis as assessed on liver biopsy (Krogsgard et al, 1984; Picchotti R et al, 1994) or by Child-Pugh classification (Pugh et al, 1973). The latter is the most commonly used means for assessing the severity of liver disease and patient survival (Table 3.1).

Although, it is generally accepted that a PPG of 12 mm. Hg. is necessary for the occurrence of variceal haemorrhage, (Viallet et al, 1975; Garcia-Tsao et al, 1985;

Table 3.1: Pugh's modification of Child's grading of the severity of cirrhosis

Clinical and biochemical measurements	Score 1 point	Score 2 points	Score 3 points
Serum bilirubin (μmol/l) (in primary biliary cirrhosis)	17 – 34 (17 – 68)	34 – 51 (68 – 110)	> 51 > 110
Prolongation of prothrombin time (seconds)	1 - 4	4 - 6	> 6
Serum albumin (gm/l)	> 35	28 – 35	< 28
Ascites	none	slight	Moderate or more
Encephalopathy grade	none	1 and 2	3 and 4

Child's – Pugh Grade A = 5 or 6 points

Child's – Pugh Grade B = 7 to 9 points

Child's – Pugh Grade C = 10 to 15 points

Minimum score = 5 points, Maximum = 15 points

(Pugh et al, 1973)

Lin et al, 1989; Rigau et al, 1989), the relationship between the severity of portal hypertension and the risk of haemorrhage remains controversial.

Portal pressure measurement

Several direct and indirect techniques have been described to evaluate portal pressure. The direct measurements are all invasive procedures involving catheterization of the portal vein. This can be performed by percutaneous transhepatic or intrasplenic puncture, by using a transjugular approach or by direct puncture of a mesenteric vein at laparoscopy. Pressures in oesophageal varices have been determined endoscopically, either by using a direct needle puncture of the varix or by using a pressure transducer that is positioned against the wall of the varix (Figure 3.4). Significant disadvantages of these techniques are their invasive nature and the potential need for anaesthesia or sedative agents, which may modify the splanchnic and systemic haemodynamics. The indirect measurement of portal venous pressure by measuring WHVP has been shown to correlate closely with directly measured portal pressure. The principle of the technique is that a catheter introduced as far as it can go (usually through the right femoral or jugular vein) into a hepatic venous radicle (i.e. wedged) reflects, the pressure at the next point of free communication with the hepatic circulation, which is the sinusoidal portal pressure. The wedged position is verified by the absence of reflux of injected contrast material and pressure measurements are taken in this wedged position (WHVP). If the catheter is now withdrawn until free blood can be obtained the catheter should be lying free in the large hepatic vein or the IVC. The pressure at this site is denoted the

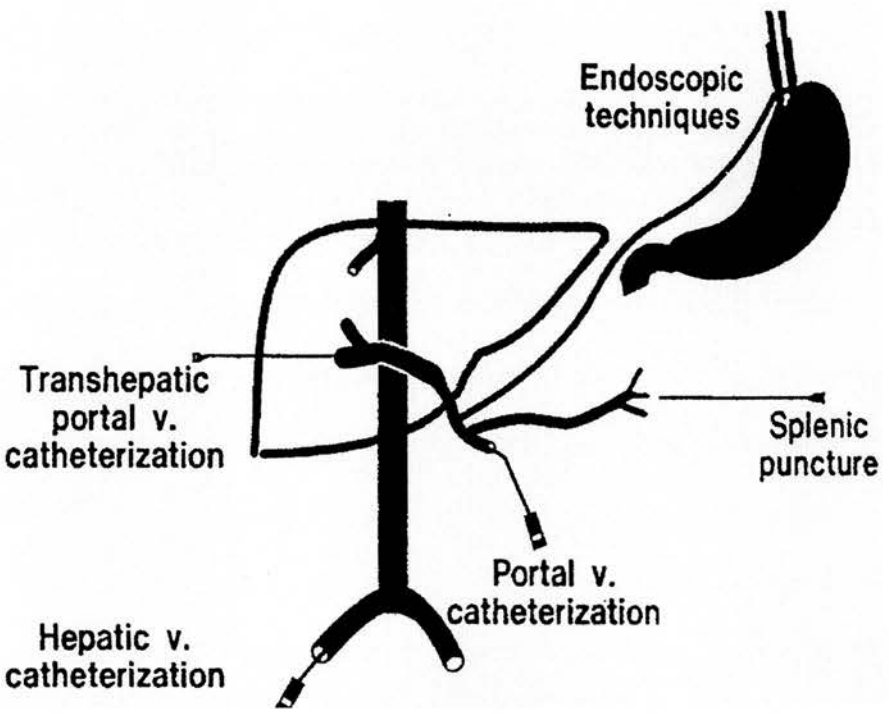

Figure 3.4: Various techniques which can be used for the measurement of Portal Pressure.

FHVP. PPG is the difference between the wedged and free pressures and reflects the gradient between portal vein and vena caval pressures.

A modification of the conventional method, the balloon catheter technique, has been described (Novak et al, 1977; Groszmann et al, 1979). With this method, the FHVP and the WHVP are obtained by inflating a balloon in the tip of the catheter. This method is the most commonly used today because it has an almost 100 percent correlation (r=0.98) with the older technique for PPG and it offers several advantages (Groszmann et al, 1979). Firstly, inflation and deflation of the balloon is much simpler than moving the catheter into and out of the wedged position. Secondly, it permits repeated pressure measurements more easily. Thirdly, injections of contrast media, which might result in a false negative increase in pressure are not needed to confirm the wedged position.

Although it is well established that WHVP is identical to portal pressure in cirrhotic dogs (Groszmann et al, 1979) and in alcoholic cirrhosis, such a good relationship in non-alcoholic liver disease has not been shown (Boyer et al, 1977; Pomier-Layrargues et al, 1985). However, two recent studies (Lin et al, 1989; Iwao et al, 1994) showed an excellent correlation between portal venous pressure and WHVP in hepatitis B- and C-related cirrhosis and suggested that the measurement of PPG reliably reflects the degree of portal hypertension in posthepatitic cirrhosis. Thus, the WHVP looks to be adequate for portal pressure measurement in most patients. There are no comparative studies in primary biliary cirrhosis, autoimmune cirrhosis or

primary sclerosing cholangitis, which, although all have a presinusoidal component, also have a significant sinusoidal component in the cirrhotic stage.

3.1.3.3 Radionuclides in hepatosplenic imaging.

The liver radionuclide scan is a map of the distribution of RE cells within the field of view. These cells are found normally particularly in the liver, spleen, bone marrow and lung. Colloid particles are removed from the circulation by phagocytosis into the RE cells and then remain there. The rate at which colloid is extracted from the blood is closely related to the number of RE cells in the tissue being perfused. Since the mass of the liver in adults is normally eight to ten times that of the spleen, the distribution of intravenously injected colloid is approximately 90 percent to the liver and 10 percent to the spleen, with a small fraction entering the bone marrow and lungs.

RE cells are distributed uniformly within the liver and the number of colloid particles injected is enough to produce a uniform distribution of radioactivity within the normal liver. Heterogenous uptake (patchy) is seen with widespread small focal lesions or diffuse parenchymal disease like cirrhosis of the liver. Splenomegaly as a result of portal hypertension shows increased colloid uptake, whereas that which enlarges due to infiltration usually shows normal or decreased uptake.

Colloid shift

The uptake of colloid in the liver, spleen and bone marrow depends upon their blood flow and extraction efficiency. Even in severe liver disease there is only a modest

loss of extraction efficiency, so the major mechanism for reduced uptake of colloid is altered blood flow. In diffuse liver disease both intrahepatic and extrahepatic shunting contribute to a reduction in effective liver blood flow. Splenic blood flow is usually increased and this together with the reduction in effective liver perfusion leads to an increase in the proportion of colloid taken up by the spleen.

3.1.3.4 SPECT

Liver scintigraphy has achieved renewed importance following the introduction of SPECT (Hill et al, 1980; Khan et al, 1981; Straus et al, 1982). Since SPECT provides high contrast images, the lesion detectibility of SPECT is superior to that of conventional imaging (Holman et al, 1979; Maublant et al, 1981; Jaszcak et al, 1982). The capability of SPECT to quantify volumes *invitro* has also been demonstrated (Kan et al, 1979; Shapiro et al, 1980; Keyes et al, 1981; Tauxe et al, 1982). Several reports have appeared in which SPECT determined volumes were compared with known phantom volumes (Shapiro et al, 1980; Tauxe et al, 1982). Others compared SPECT estimates of liver volume with those calculated from body weight and height (Kan et al, 1979). Accurate and reproducible results in volume measurement have been achieved by this technology.

3.2 STUDIES RELATING TO SPLENOMEGALY IN PORTAL HYPERTENSION DUE TO HEPATIC CIRRHOSIS.

3.2.1 Splenomegaly; Clinical aspects

3.2.1.1 Background

Splenomegaly is an early sign of portal hypertension and is a useful pointer towards making a diagnosis of portal hypertension in cirrhosis of the liver (Sherlock S, 1989). However, the spleen has to enlarge significantly before becoming palpable making clinical palpability an insensitive diagnostic tool for early splenomegaly in portal hypertension. Ultrasonography is a useful, non-invasive and widely available technique for measuring spleen size *invivo* (McGaham and Goldberg, 1998) but its role in difficult clinical situations like obesity and ascites is not clear.

Furthermore, the pathogenesis of splenomegaly in cirrhosis is multifactorial (Dumont et al, 1974) and the relationship of spleen size to the development of GOV and bleeding from them and severity of liver disease has not been thoroughly investigated (also see sections 3.1-3.1.1.4, 3.1.2, 3.1.3.1).

3.2.1.2 Aims

The aims of the present study were to retrospectively compare the detection of splenomegaly clinically with spleen size measured by imaging in patients suffering from cirrhosis of the liver and also to determine the value of abdominal ultrasound in assessing spleen size. The relationship of spleen size to the development of bleeding GOV and severity of liver disease has also been investigated.

3.2.1.3 Patients and Methods

Case notes of 111 patients who died of liver cirrhosis, at the Royal Infirmary of Edinburgh, Scotland, during a 10 years period (1988-1998) and underwent autopsy were analyzed. Aetiology of liver cirrhosis, age and sex of patients, clinical palpability of the spleen, the presence of GOV, ascites and autopsy spleen weight were recorded. Spleen size as assessed by clinical palpation during the last admission of the patient prior to death was noted and ultrasonically determined length of spleen shortly before death where available was recorded. There was a time gap of less than four weeks between clinical and ultrasound examination of each patient. Severity of liver disease as assessed by Child's grade (Pugh et al, 1973) was also noted. The relationship between clinical palpability, ultrasonic dimensions and autopsy spleen weight was studied. The presence of bleeding GOV and Child's score was also correlated with spleen size.

Statistical analysis: This was done using SPSS software. Non-parametric Mann-Whitney 'u' test and Kruskal-Wallis test were used to compare mean spleen weight of palpable and non-palpable spleens in the absence of ascites and mean weight of

palpable spleens in the presence of moderate, small or no ascites respectively. Pearson's chi-square test was used for comparison of variceal size and bleeding episodes in patients with and without splenomegaly (Table 3.3). Independent samples 't' test was used to compare the mean spleen weight in patients with large and moderate varices versus those with small or no varices.

3.2.1.4 Results:

The mean age of patients at death was 60.5 years (median 61 years; range 16-90 years), the male/female ratio being 68/43 patients (61.26/38.74%). Aetiology of chronic liver disease is shown in Table 3.2. Most patients had Child's grade C cirrhosis, being present in 57 (51.3%) as compared to 13 (11.7%) and 41 (37.0%) of Child's grade A and B respectively. Eighty-one patients (73%) had ascites (mild 12, moderate 34, severe 35 patients). Seventy patients (63.1%) had varices documented at upper gastrointestinal endoscopy prior to death (large 28, moderate 26 and small 16 patients).

Splenomegaly at autopsy, which was taken as spleen weight greater than 150 gms. was observed in 93 (83.8%) patients. Figure 3.5 shows the relationship between clinical palpability and autopsy spleen weight in the absence of ascites, showing that palpable spleens are bigger than impalpable spleens. Comparing spleen weights in all patients, including those with ascites, spleens which were palpable (571.7±81.0 gms.) were twice as heavy at autopsy than those which were not palpable (281±16.2 gms. p=0.003).

Comparison of autopsy spleen weights (mean weight[gm.] ± s.e.m) in patients having palpable spleens in the presence of moderate and small amount and those without ascites is shown in Figure 3.6. There was only one patient with palpable spleen in the severe ascites group, making valid statistical analysis difficult for this group and therefore was not included in the analysis.

Although spleen weights in Child's grade A&B vs. C were not significantly different (375±39.7 vs.332.5± 30.6 gms. $p>0.05$), it was less often palpable in Child's C patients ($p=0.002$). Patients who had splenomegaly at autopsy had larger varices and a history of GOV bleeds as compared to those with normal spleen weight (Table 3.3). Autopsy spleen weight was greater in patients who had large to moderate sized varices at endoscopy as compared to those who had smaller varices and had not bled from them (Table 3.4).

Abdominal ultrasonography carried out on overnight fasted patients showed that autopsy spleen weight had a strong correlation with ultrasonically determined length of the organ shortly before death ($r=0.78$, $p=0.001$; Figure 3.7).

Table: 3.2 Aetiology of chronic liver disease in 111 patients with portal hypertension.

AETIOLOGY	Number = n (%)
Alcohol	64 (57.7)
Primary Biliary Cirrhosis	13 (11.7)
Cryptogenic Cirrhosis	8 (7.2)
Haemochromatosis	4 (3.6)
Post Hepatitic Cirrhosis	2 (1.8)
Primary Sclerosing Cholangitis	1 (0.9)
Portal Vein Thrombosis + Cirrhosis	1 (0.9)
Alpha 1 Anti-trypsin Deficiency	1 (0.9)
Idiopathic Portal Hypertension	1 (0.9)
Secondary Biliary Cirrhosis	1 (0.9)
Not recorded	15 (13.5)
Total	111 (100)

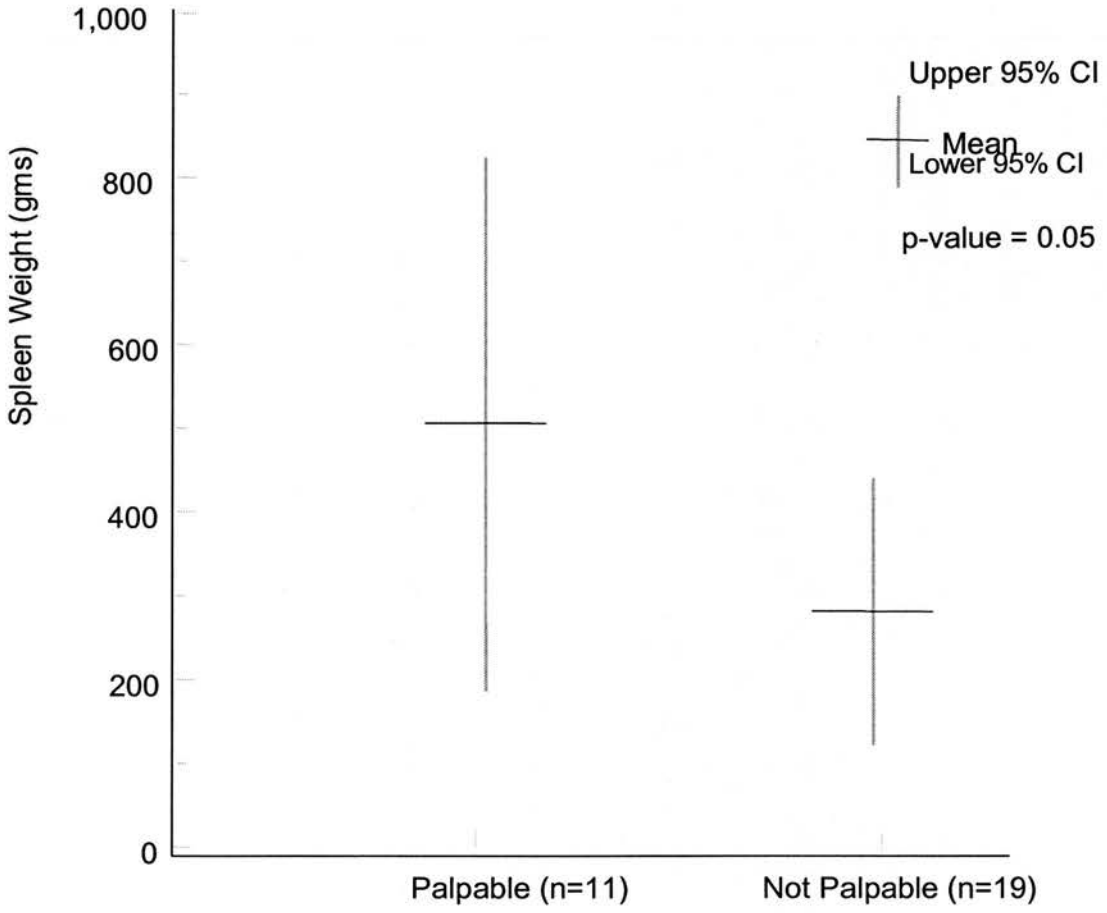

Figure: 3.5 The comparison of spleen weight (gms.) between palpable and not palpable spleen in the absence of ascites.

Table 3.3 Comparison of variceal size and history of GI bleeds between patients with enlarged and normal sized (autopsy weight <150 gms.) spleens.

Factors	Spleen (n)		p-value
	Enlarged	Normal	
Variceal size			
• Large & Moderate	50	5	
• Small and Absent	45	11	0.06
Bleed			
• Yes	63	7	
• No	31	10	0.04

Table: 3.4 Comparison of autopsy spleen weight (gms.) between patients with large and moderate sized varices vs those with small or absent varices.

Factor	Variceal Size		*p-value*
	Large & Moderate (n=55)	**Small & Absent (n=56)**	
Spleen weight (gms.)	405.9 $\pm$ 252.2	298.7 $\pm$ 225.3	0.02

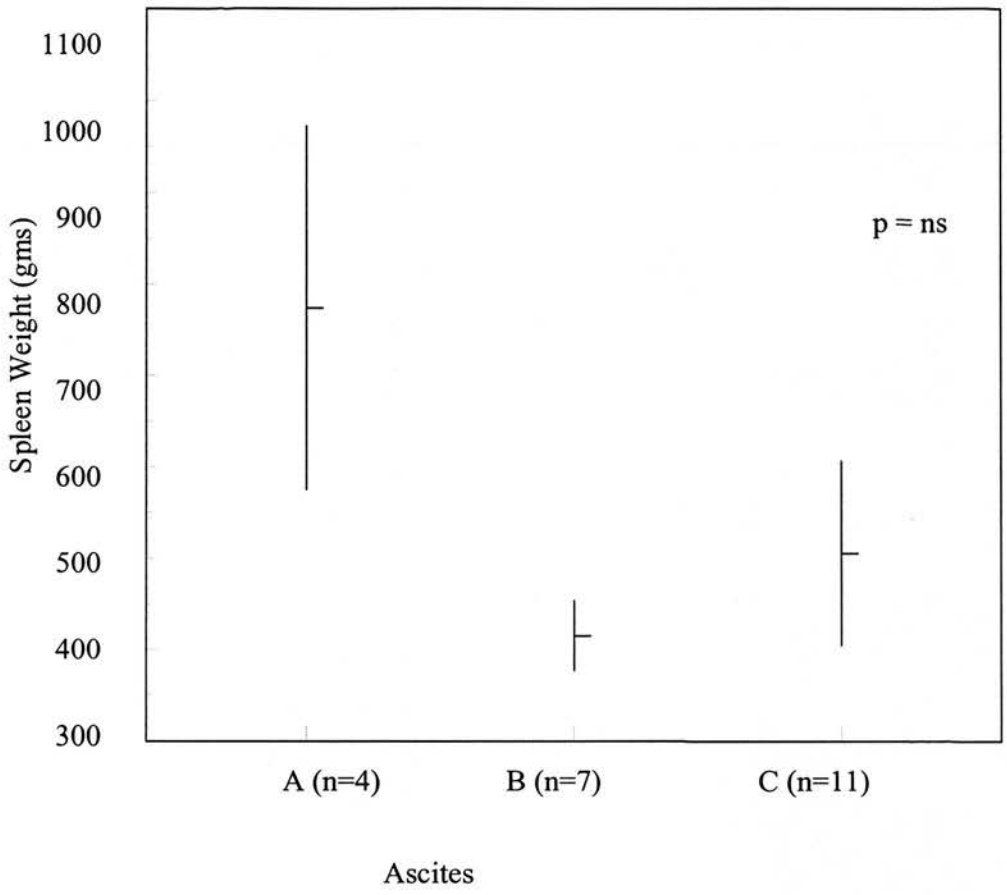

Figure 3.6: Mean weight of palpable spleens (gms) ± s.e.m. in patients with (A) moderate ascites (B) small ascites and (C) without ascites.

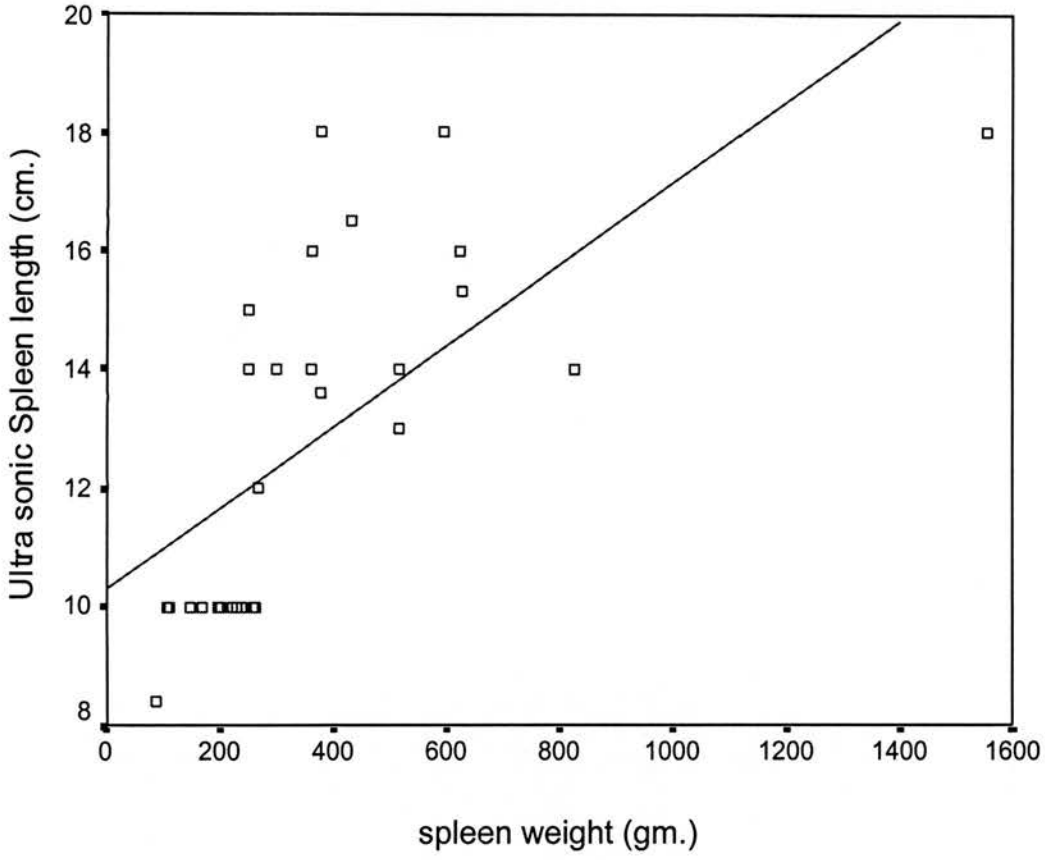

Figure 3.7: The relationship of Spleen length (cm.) vs. Spleen weight (gm.)

3.2.1.5 Discussion

In a retrospective analysis of case records of 111 patients suffering from cirrhosis of the liver who died during a 10 years period (1988-1998) and underwent autopsy, the correlation of autopsy spleen weight to clinical palpability, severity of liver disease and its impact on palpability has been investigated. Clinical correlates of portal hypertension like the development of GOV and bleeding from these vessels have also been correlated to spleen size.

Although it is observed from the present data that spleens which were heavier at autopsy were more often palpable in life, there was a considerable overlap of spleen sizes in the palpable and non-palpable spleen groups (Figure 3.5), impairing the sensitivity of palpation as a clinical tool in diagnotics. This is in agreement with the findings of Ratnoff and Patek (1942) who analyzed autopsy data on alcoholic cirrhotic patients and observed a considerable overlap in liver and spleen weights between palpable and non-palpable groups.

Furthermore, the presence of ascites makes clinical palpation more difficult and the spleen has to enlarge much more before becoming palpable in the presence of ascites when compared to patients without ascites, and there is also a considerable overlap of spleen weight between groups (Figure 3.6). Hence, there is a need for a laboratory investigational tool to measure spleen size accurately.

Abdominal ultrasonography has been used for a number of years with a steady improvement in the quality of imaging leading to better sensitivity and specificity in

diagnosis of liver disease (Weill, 1996). We have used it in our patients to measure maximum length of spleen and correlated it with autopsy spleen weight. The good correlation of autopsy spleen weight and ultrasonically determined length (Figure 3.7) indicates that abdominal ultrasonography can be used to estimate spleen size accurately, in clinical practice. Zhang and Lewis (1987) compared radio-isotopically determined size of spleen with spleen volume determined following splenectomy and found a good correlation. We have used spleen weight as a measure of actual spleen size and compared it with ultrasonographically determined spleen dimensions, which is a much simpler and non-invasive technique to determine spleen size compared to radionuclide studies.

The pathogenesis of splenomegaly in hepatic cirrhosis is multifactorial, but haemodynamic factors could be the most important determinants of spleen size. The presence of larger varices and more frequent bleeds in patients with larger spleens suggests a possible role for increasing portal pressures and splanchnic blood flow secondary to vasodilatation in cirrhosis of liver in producing splenomegaly. The good correlation of spleen size with portal vein blood flow volume, as shown subsequently (Section 3, Figure 3.9), and the finding here of a relationship of increasing spleen size with larger varices and a history of bleeds suggests the importance of portal haemodynamic factors in producing splenomegaly. However, it is not clear from the results of the present investigation if splenomegaly leads to these haemodynamic changes in hepatic cirrhosis or develops as a consequence of them. Since these complications of portal hypertension are important clinical consequences of disease, spleen size measured non-invasively by ultrasonography

would identify those patients with larger spleens most at risk of large varices and variceal haemorrhage.

3.2.1.6 Conclusions

In conclusion, the present study highlights the problems in clinical diagnosis of splenomegaly. Palpation of the spleen is of limited value in diagnosing early splenomegaly and the presence of ascites further impairs palpability. Abdominal ultrasound assesses spleen size accurately and allows splenomegaly to be identified easily. Spleen size correlates well with the presence of larger GOV and of bleeding from theseGOV, but no correlation with Child-Pugh's grade has been observed.

3.2.2 Measurement of spleen size and its clinical correlates in hepatic cirrhosis.

3.2.2.1 Background

Splenomegaly is a cardinal feature of hepatic cirrhosis complicated by portal hypertension and is observed in a large proportion of such patients. Mechanisms whereby it is produced continue to be debated. Attempts to assess the relationship of spleen size to factors such as portal pressure, portal haemodynamics and features of hypersplenism require reliable methods of measuring spleen size, like ultrasonography, radionuclide imaging, CT scanning and MRI. Few studies have compared these methods to one another leading to uncertainty in interpreting the results of studies using different methods.

3.2.2.2 Aims

To compare ultrasonography with radionuclide imaging in measuring spleen size in patients suffering from cirrhosis of the liver and portal hypertension. Spleen size has also been related to the peripheral blood features of hypersplenism.

Spleen volume has been compared with portal haemodynamic measurements to see whether these might explain the splenomegaly of portal hypertension.

3.2.2.3 Patients and Methods

Twenty-five patients (sixteen males) with hepatic cirrhosis and portal hypertension were studied prospectively. Ethical approval for the study was obtained from the Lothian regional ethics committee. Cirrhosis was diagnosed by liver biopsy in seven patients; of these seven patients, portal hypertension was diagnosed by WHVP in 3 patients and finding of GOV in 4 patients. Cirrhosis was diagnosed by clinical features, abnormal liver function tests and ultrasonography in 18 patients; of these patients, portal hypertension was diagnosed by WHVP measurement in 9, from the presence of GOV in 7, and splenomegaly in 2 patients. Fifteen patients had alcoholic cirrhosis, 5 had primary biliary cirrhosis, 2 had autoimmune hepatitis and cirrhosis, 1 had hepatitis C virus liver disease and 2 had cryptogenic cirrhosis.

Ultrasound imaging was carried out after an overnight fast using an Acuson 128 instrument with a 3.5 MHz transducer. The abdomen was scanned initially, and the shape, size and the texture of the liver was noted, together with the presence or absence of ascites. The spleen was then examined to measure spleen size. The greatest length, transverse diameter and thickness at the hilum were measured. These were then multiplied together and a further factor of 0.6 was included to obtain an approximation of the volume. The portal vein was examined during quiet respiration with the patient supine and in the right anterior oblique position. The cross-sectional area was measured immediately below the bifurcation on three occasions, and a mean value was obtained. Mean averaged velocity was then measured using pulsed doppler on three occasions and a mean value was calculated. An approximation of the flow volume was then calculated by multiplying the average cross-sectional area

(cm^2), the mean time averaged velocity (cm/sec), and a factor of 60 to obtain flow volume in ml/min.

Radionuclide imaging of liver and spleen was carried out on the same day as ultrasonography after administration of 140 MBq of $^{99}Tc^m$ albumin colloid and SPECT was performed using a GE 400 AT gamma camera and a Seimens Microdelta Plecs Computer. Data were collected in 64 views, each 64×64 pixels, over an arc of 360 degrees. Transverse slices were constructed by back projection using a Butterworth filter of order 3 with a cut-off at 0.5 of the Nyquist frequency. Liver and spleen volumes were determined by a semi-automatic method whereby the operator viewed the transverse slices as a movie sequence, first determining which slices contained images of the organ concerned and then drawing a rectangular ROI that enclosed the entire organ. Each frame was then smoothed twice using a nine-point weighted filter to reduce statistical noise, and the volume of the cuboid described by the rectangular ROI and the selected frames were searched to determine the maximum value. The operator was shown each frame in turn and given the opportunity to redraw the ROI of the frame to exclude intruding structures (e.g. liver in the spleen ROI). Then, for each frame, all pixels above a threshold of 50% of the maximum were summed. The 50% threshold was determined by calibrating the pixel size, then imaging phantoms with volumes in the range 400-1000 ml. at various thresholds to determine which gave the most accurate result. Percentage uptake of the total injected dose of colloid by the liver and spleen, the L/S ratio and uptake of radio-isotope by the spleen/unit volume of the organ was

calculated and correlated with spleen size and severity of liver disease as assessed by Child's score (Pugh et al, 1973).

Sample trace of hepatosplenic SPECT in one of the patients is shown in appendix V.

The WHVP and FHVP were measured at hepatic vein catheterization using a balloon occlusion sidewinder catheter (Cordis) and the portal pressure gradient (PPG) calculated (PPG =WHVP-FHVP) in fifteen of the twenty-five patients.

Sample trace of FHVP and WHVP from one of the patients is shown in appendixVI.

Measurement of Azygos vein blood flow

This was carried out following the insertion of an azygos catheter (Webster Lab Inc) into the azygos vein under fluoroscopic screening in seven of the twenty-five patients only. The electric integrity of the thermistor was checked prior to insertion into the patient by a custom built circuit tester made by the Department of Medical Physics, Royal Infirmary of Edinburgh, Scotland..

Estimation of azygos vein blood flow was made by the reverse thermodilution technique (Hayes et al, 1992) using 5% dextrose at room temperature at 50 ml./min. as injectate. The syringe pump (Harvard model 22 modified to meet BS5724 Standard) was used to deliver the dextrose accurately into the distal lumen of the azygos catheter. The electrical connection of the catheter was connected to a custom- built IBM model PS2-286 microcomputer interface to give real time values for blood flow.

The haemodynamic and imaging studies were done within the same week for each patient.

Statistical analysis: Data were analyzed using Statistical Package for Social Scientists (SPSS software).Linear correlations between various variables were calculated and scatter plots constructed. A $p<0.05$ was taken as significant (two tail test of significance).

3.2.2.4 Results

There was a close correlation between ultrasonographically and radio-isotopically determined spleen volume ($r=0.95$, $p<0.001$; Figure 3.8). There was a good correlation between the spleen volume determined ultrasonically and the portal vein blood flow volume (ml./min.) ($r=0.57$, $p<0.008$; Figure 3.9).

No significant correlation was found between spleen size, PPG, portal vein blood flow velocity or the azygos vein blood flow. The radionuclide uptake of the spleen increased as the volume of the spleen increased ($r=0.43$, $p<0.04$; Figure 3.10) but no correlation was found between liver and spleen uptake of radioisotope. Spleen volume was negatively correlated with the WBC count ($r= -0.43$, $p=0.05$; Figure 3.11), but no correlation between the spleen volume and the haemoglobin concentration or platelet count was observed. The phagocytic activity of the spleen as evidenced by radionuclide uptake was negatively correlated with the haemoglobin concentration ($r= -0.48$, $p<0.04$; Figure 3.12) and WBC count ($r= -0.46$, $p<0.04$; Figure 3.13) but no correlation was found between spleen radionuclide uptake and platelet count.

Since multiple variables have been correlated with each other in this analysis assessing simple linear correlations, there is a potential for a type 1 error being high and the correlations which are close to a significance value of < 0.05 may actually be non-significant.

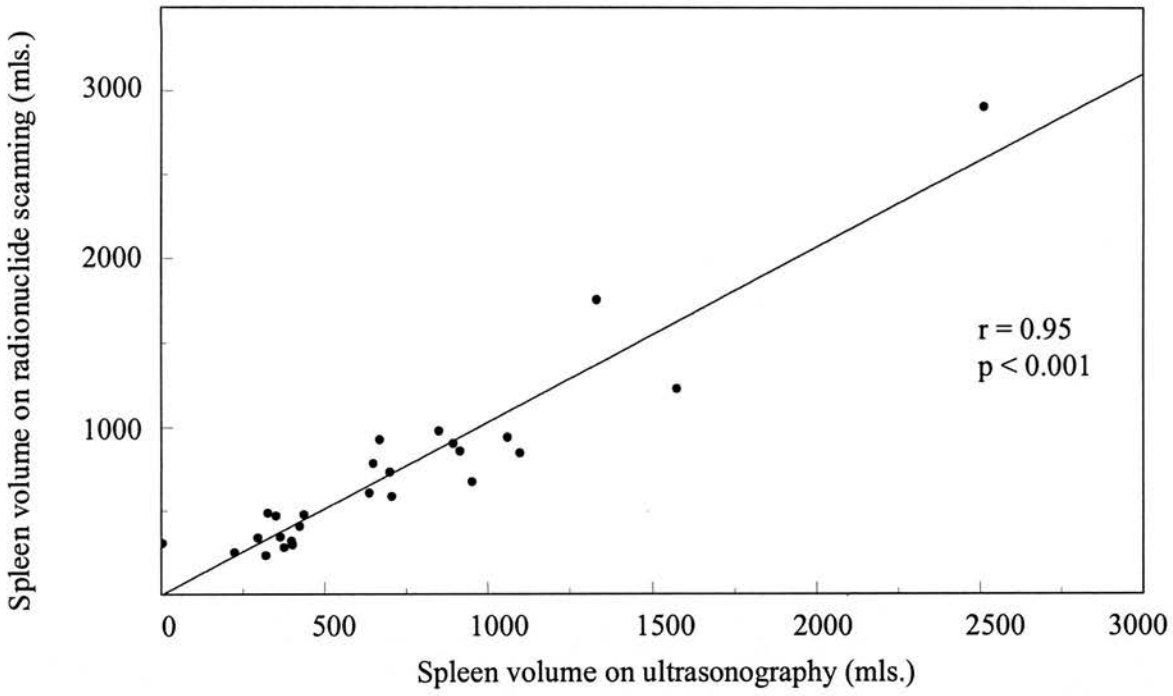

Figure 3.8: The relationship of the spleen volume measured by ultrasonography (mls.) to that measured by radionuclide imaging(mls.).

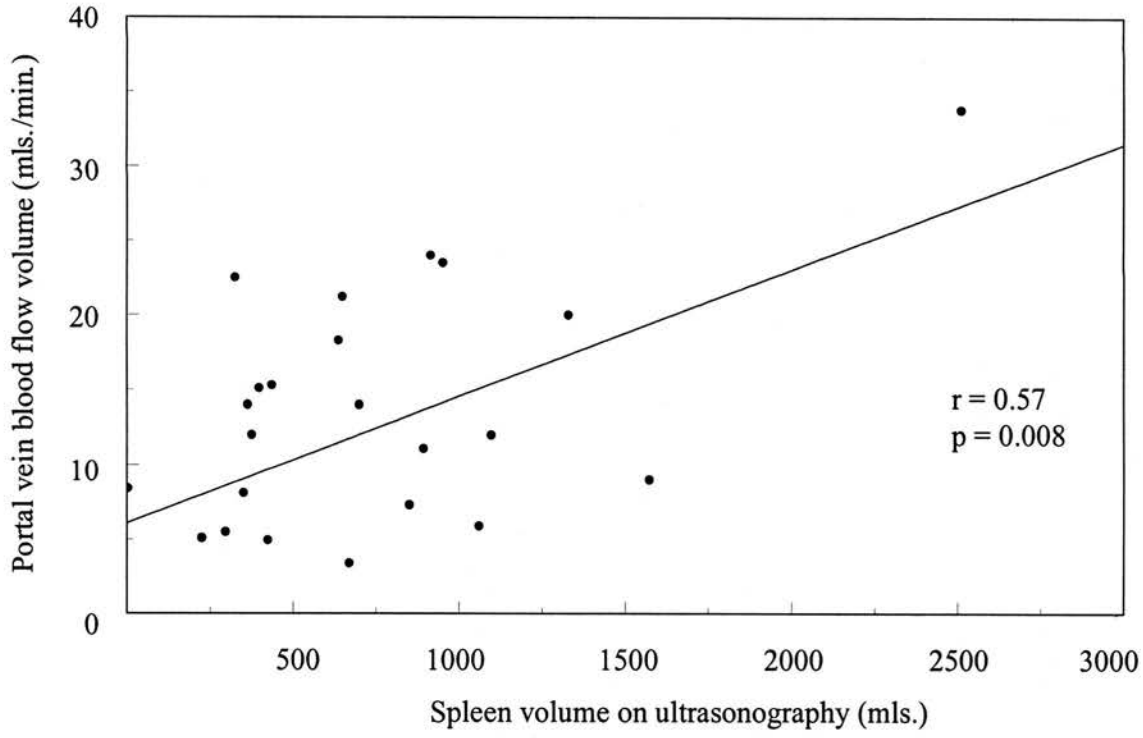

Figure 3.9: The relationship of the spleen volume measured by ultrasonography (mls.) to the portal vein blood flow volume (mls./min.).

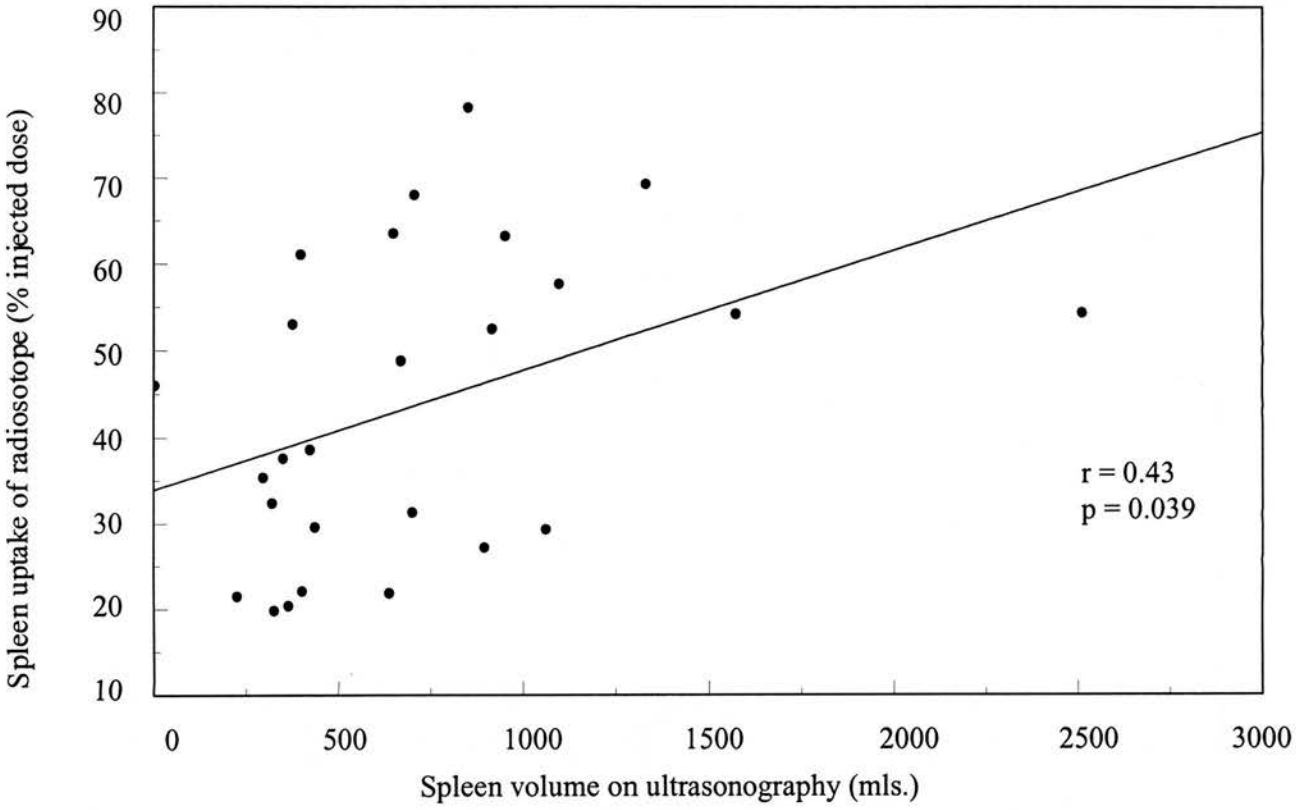

Figure 3.10: The relationship of spleen uptake of radioisotope ($^{99}Tc^{m}$ albumin colloid) by the spleen to the ultrasonically determined volume of spleen (mls.).

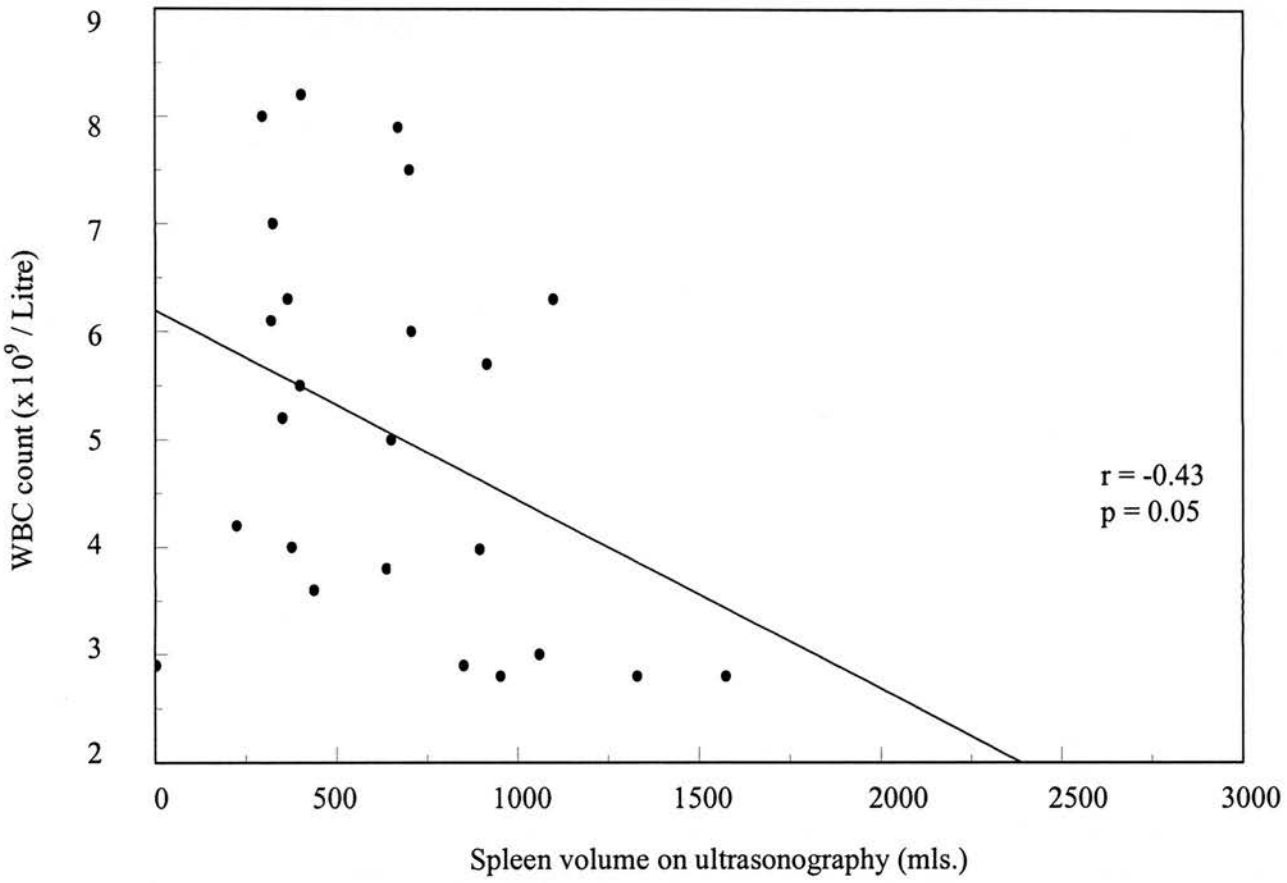

Figure 3.11: The relationship of peripheral total WBC count ($x10^9$ / Litre) to the volume of the spleen determined at ultrasonography (mls.).

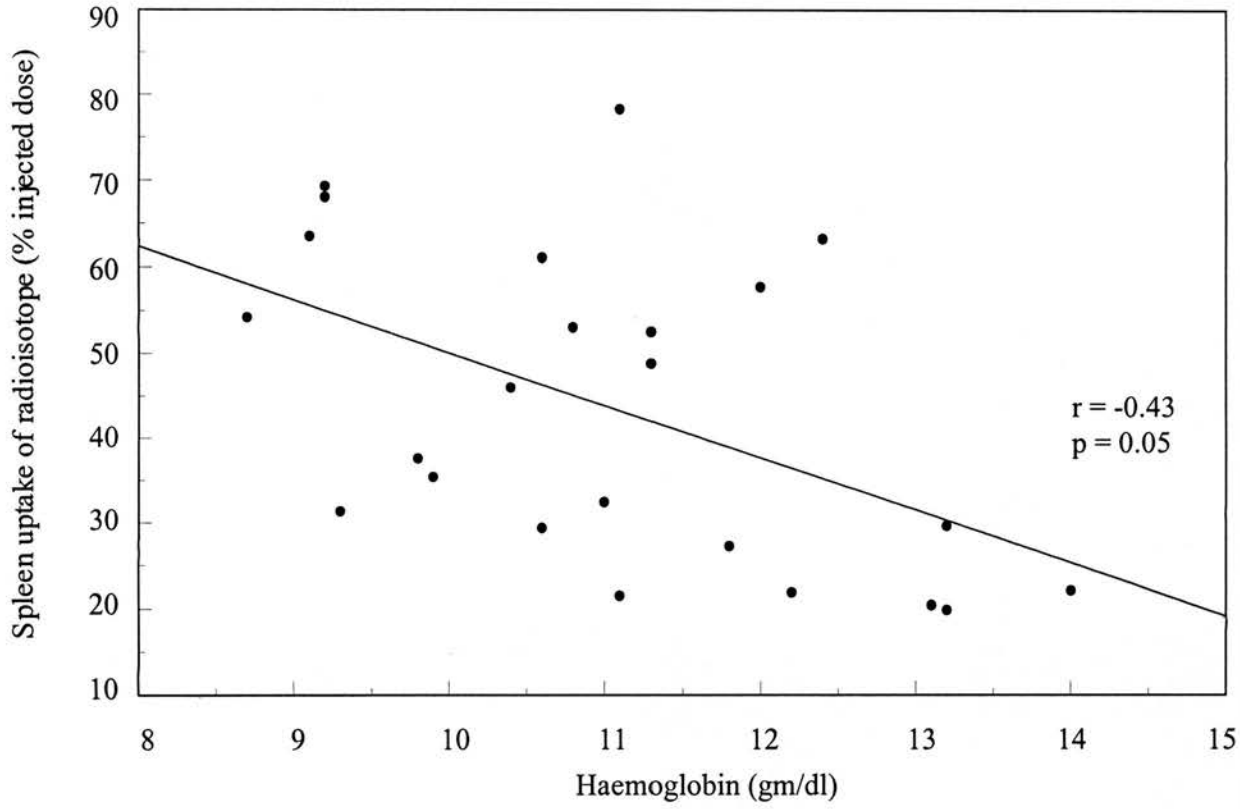

Figure 3.12: The relationship of Haemoglobin concentration (gm/dl) and spleen uptake of radioisotope (% injected dose).

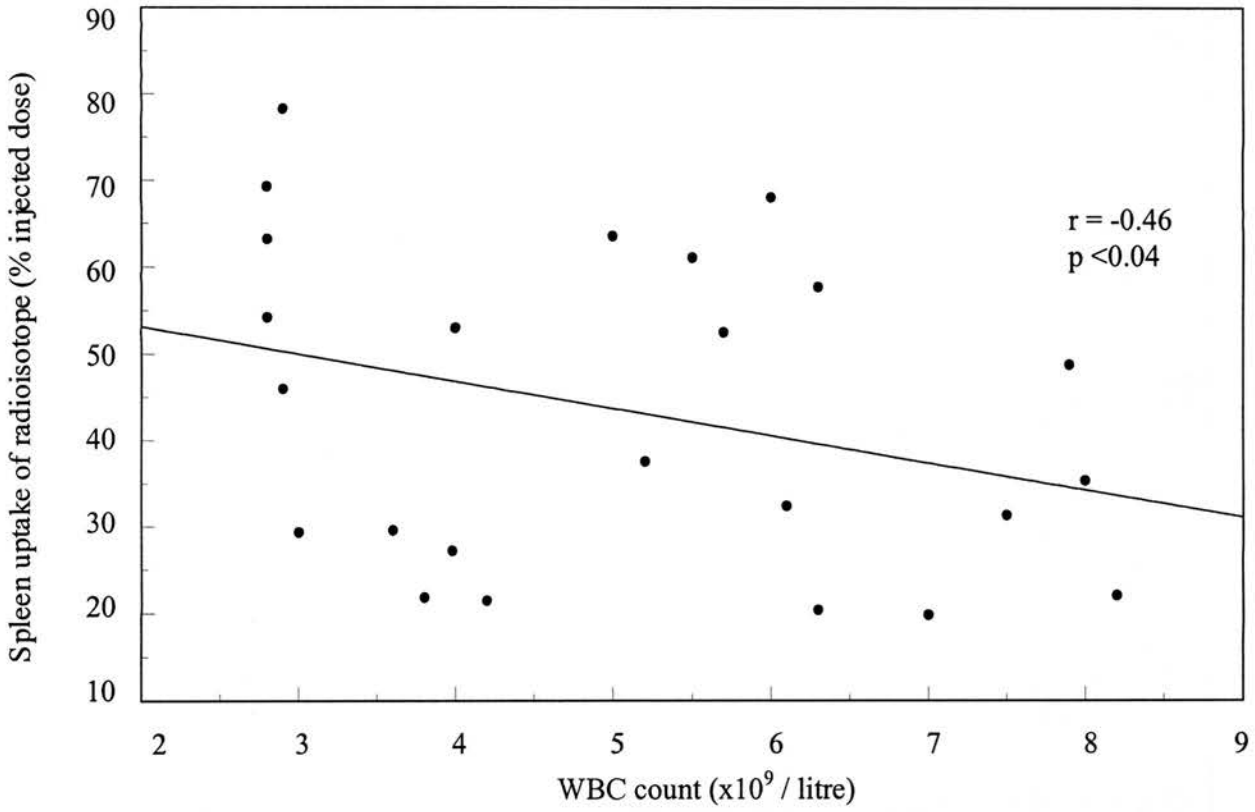

Figure 3.13: The relationship of total WBC (x10^9 / litre) to spleen uptake of radioisotope (% injected dose).

3.2.2.5 Discussion

The present investigation has attempted to explain the cause of splenomegaly in cirrhosis of the liver and also investigated the relationship of haematological parameters of hypersplenism to spleen size and radionuclide uptake. The close correlation of spleen volume as determined by ultrasonography and radionuclide uptake in splenomegaly due to cirrhosis of the liver shows that these methods of measuring spleen size are comparable. Zhang and Lewis (1987) compared ultrasonography and scintigraphy in measuring spleen size in haematological disorders. Ultrasonography measures total spleen mass by assessing volume, whereas scintigraphy depends upon organ blood flow and functionally active reticuloendothelial tissue to show splenic dimensions. The good comparability of these two methods of measuring spleen size implies that the two methods can be used alternatively. It also means that the phagocytic cell mass and blood flow in the spleen increases as the spleen enlarges as is seen in splenomegaly of haematological disorders. Thus, RE cell hyperplasia must be an important factor in producing splenomegaly.

The increase of radionuclide uptake by the spleen is not related to and thereby possibly not a consequence of diminishing liver uptake of radionuclide because there was no correlation between liver and spleen colloid uptake on scintigraphic measurement in our patients. This conclusion, however, is not in agreement with the results of previous studies (Jacob et al, 1963). This difference in observation may be accounted for by differences in the patient populations studied in the two investigations.

A number of factors are important in the production of hypersplenism in cirrhosis of the liver. Hypersplenism is defined as one or more of thrombocytopenia, leucopenia and sometimes anaemia with enlargement of the spleen and a normal or hypercellular bone marrow. The formed elements of blood are suppressed to varying degrees and there is recovery after splenectomy (Tumen, 1970, see also section 3.1.1.3). Various mechanisms to account for the development of hypersplenism in the splenomegaly of portal hypertension in hepatic cirrhosis have been proposed (Doan, 1949). These include increased pooling of blood cells in the spleen, increased destruction of blood cells in the spleen, dilutional effects of an increased blood volume and humoral factors (Schaffner et al, 1985). Thrombocytopenia is the most common manifestation of hypersplenism in cirrhosis and portal hypertension and previous studies have quoted a negative correlation between spleen size and platelet count (Al-Khishen et al, 1985). The results of the present investigation do not show any correlation between the platelet count and ultrasonic spleen volume or phagocytic activity of the spleen as reflected in the radionuclide uptake scan suggesting that other factors than spleen size and phagocytic activity must also be important in determining the circulating platelet levels. These could include the presence and amount of immunoglobulin on the platelet surface (Barrison et al, 1982). The results of the present study show a negative correlation of the WBC count to spleen size measured by ultrasonography (Figure 3.11) and radionuclide uptake of spleen (Figure 3.13). The spleen size and increased phagocytic activity of RE cells reflected on radionuclide scan may therefore be more important in relation to leukopenia in hypersplenism. The haemoglobin levels were inversely related to

the radioisotope uptake by the spleen (Figure 3.12). This suggests the importance of RE cell activity in the spleen in determining red blood cell turnover.

Splenomegaly in cirrhosis is accompanied by important haemodynamic changes, and splenomegaly in cirrhotic patients may in fact result from these pathophysiological changes (Blendis et al, 1969; Merkel et al, 1985). The strong positive correlation of the portal vein cross-sectional area and portal blood flow volumes with spleen size points to a close relation between circulatory factors and splenomegaly, but does not indicate which is the cause and which is effect. Furthermore, the lack of correlation of spleen size with portal vein pressure and collateral vein blood flow through the azygos vein implies a complex rather than a simple relationship. One interpretation of these data might be that spleen size is determined, at least early in the disease process by portal congestion, thereby explaining the close association with portal vein blood flow volume. As the liver disease progresses, however, congestion does not increase because decompression via portal collaterals measured by azygos blood flow occurs. Indeed, porta-systemic shunting through the azygos system increases significantly, as the liver disease advances (Braillon et al, 1986).

It is interesting that although a number of studies have found doppler ultrasound measurements valuable in predicting events or drug responses in patients with portal hypertension, it is the congestive index (ratio of portal vein cross-sectional area and velocity) rather than cross-sectional area alone that is clinically relevant, again

suggesting the complex nature of the relationship between haemodynamic factors and splenomegaly (Moriyasu et al, 1986).

Portal hypertension and its consequences develop slowly over years, and there is likely to be a considerable temporal separation between the development of established portal hypertension and the mechanisms causing splenomegaly. The patients studied in the present investigation had well established portal hypertension, often with oesophageal varices which are a late development in this condition, and haemodynamic measurements at this stage may not reflect changes present during the development of splenomegaly.

3.2.2.6 Conclusions

In conclusion, the results point towards the general importance of circulatory factors in splenomegaly of hepatic cirrhosis but cannot delineate primary or secondary mechanisms. Hypersplenism in portal hypertension affects the different formed elements of the blood to different degrees and these studies point to splenomegaly and its reticuloendothelial effects as being more important in leucopenia and anaemia than in thrombocytopenia where extrasplenic factors seem to be more important.

3.2.3 The role of SPECT in assessing liver function in hepatic cirrhosis.

3.2.3.1 Background

Splenomegaly is an early and important sign of portal hypertension due to cirrhosis of the liver. If the spleen is not palpable and is not enlarged on imaging, the diagnosis of portal hypertension is questionable (Sherlock S, 1989). However, the pathogenetic mechanisms whereby splenomegaly occurs continue to be debated and the relationship of spleen size, R-E cell phagocytic function and vascularity of the organ to the severity of liver disease has seldom been studied (Alstead et al, 1987). Conventional two-dimensional scintigraphy has been used in the diagnosis (Millete et al, 1973) and evaluation of disease severity in cirrhosis of liver (Castell and Johnson, 1966; Beckerman and Gottschalk, 1971). However, improvements in imaging techniques have led to its replacement by other methods such as ultrasonography (Koga and Moikawa, 1975; Niederan et al, 1983), CT scanning (Heymsfield et al, 1979), SPECT (Straus et al, 1984; Mut et al, 1988) and MRI (Williams et al, 1985). SPECT allows a three dimensional assessment of the liver and spleen (Kan and Hopkins, 1979, see also section 3.1.3.4). Whereas SPECT of the liver has been shown to give useful information about disease severity, the place of spleen SPECT has been investigated in only a small number of patients (Alstead et al, 1987) and its place in this regard is not clear.

3.2.3.2 Aim

To determine the value of SPECT of spleen in assessing severity of liver disease in hepatic cirrhosis.

3.2.3.3 Patients and Methods

$^{99}Tc^{m}$ –albumin colloid was used to perform hepato-splenic SPECT in twenty-five patients (sixteen males) suffering from cirrhosis of the liver of varied aetiology. Cirrhosis was diagnosed on the basis of histology in seven patients. In others, cirrhosis was diagnosed by clinical features, abnormal liver function tests and ultrasonography.Ultrasound imaging was carried out after an overnight fast using an Acuson 128 with a 3.5 MHz transducer. The abdomen was scanned initially and the shape, size and texture of the liver was noted, together with the presence or absence of ascites. The spleen was then examined to measure spleen size as an indicator of portal hypertension. The portal vein was examined during quiet respiration with the patient supine and in the right anterior oblique position; the cross-sectional area was measured immediately below the bifurcation on three occasions, and a mean value was obtained (see also section 3.2.2.3). Radionuclide imaging of liver and spleen was carried out after administration of 140 MBq of $^{99}Tc^{m}$ albumin colloid and SPECT was performed using a GE 400 AT gamma camera and a Seimens Microdelta Plecs Computer (see also section 3.2.2.3).

Statistical analysis: This was done using SPSS software. ANOVA test was applied to calculate the significance between groups of patients in Table 3.5 and simple linear correlation done for variables in Figure 3.14.

3.2.3.4 Results

Twenty-five patients were studied. Fifteen had alcoholic cirrhosis, 5 had primary biliary cirrhosis, 2 had autoimmune hepatitis and cirrhosis, 1 had hepatitis C virus-induced liver disease and 2 had cryptogenic cirrhosis.

Table 3.5 shows the relation of spleen uptake of colloid/unit volume of spleen (% uptake of injected dose of isotope/ml) and L/S ratio to disease severity. The spleen seems to enlarge with worsening of liver disease from Child's grade A to B, but then fails to enlarge any further and may actually regress in size with the development of collaterals as liver disease worsens to Child's grade C.

Although total uptake of radio-colloid (% injected dose) is not significantly different between groups, the spleen uptake/unit volume is higher in Child's grade C patients implying more vascularity per unit of tissue. L/S ratio diminishes with worsening liver function which again suggests decrease in liver blood flow and also phagocytic cell mass relative to the spleen.

Figure 3.10 shows the positive relationship of spleen uptake of colloid (% of injected dose) to volume of spleen as assessed by radionuclide study ($r=0.43$, $p=0.039$). Spleen uptake of colloid/unit volume showed statistically significant negative association with ultrasonically determined volume of spleen ($r=0.46$, $p<0.04$: Figure 3.14), implying no net increase in vascularity of the organ/unit of tissue.

Table: 3.5: The relation of spleen uptake of colloid/unit volume of spleen (% of injected dose of isotope/ml) and L/S ratio to Child's grade of liver disease.

	Child Grade			p-value
	A(n=10)	B(n=10)	C(n=5)	
Spleen Uptake (% of injected dose of isotope)	41.53 ± 19.54	41.23 ± 18.09	54.15 ± 15.79	0.374
Spleen Volume (mls)	590 ± 272	777 ± 447	444 ± 221	0.26
Spleen Uptake per unit volume (% of injected dose of isotope/ml)	0.084± 0.056	0.059 ± 0.019	0.135 ± 0.041	0.035 A vs B } ns
L/S ratio	1.18 ± 0.58	0.81 ± 0.56	0.35 ± 0.079	0.049 A vs B}ns B vs C }ns

Results are expressed as mean ± SD

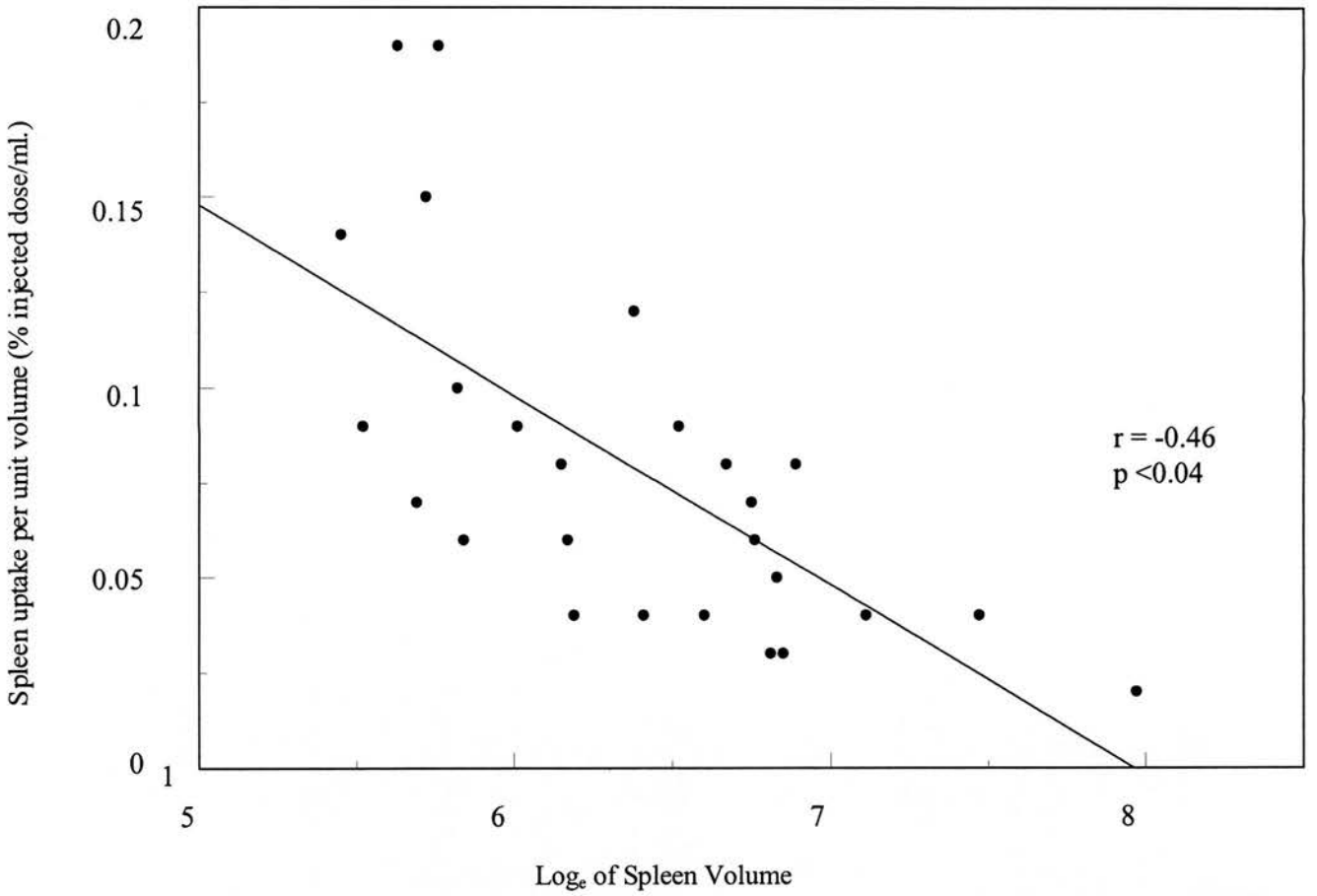

Figure 3.14: The relationship of spleen uptake of radioisotope per unit volume (% injected dose/ml.) and $\log_e$ of spleen volume.

3.2.3.5 Discussion

SPECT of the spleen, although not used frequently in diagnosis, is a non-invasive and safe procedure which gives reliable results (Meek et al, 1984). The amount of radio-pharmaceutical administered exposes the patient to a minimum dose of radiation. Investigations relating to liver and spleen scintigraphy have mostly used Sulphur or Tin Colloid of $^{99}Tc^{m}$ (Parkin et al, 1983; McLaren et al, 1985; Bolton, et al, 1988; Rutland and Que, 1995). However, there are problems in the preperation of these colloids which are overcome with albumin colloid and this exposes the operator to a minimum of radiation dose (Saha et al, 1986). We have used $^{99}Tc^{m}$ albumin colloid to accomplish hepato-splenic SPECT in our patients.

Although larger spleens take up more radio-colloid, this is not a reflection of increasing vascularity per unit of tissue, as the uptake/unit volume decreases with increasing spleen size (Figure 3.14). This suggests that splenic enlargement in portal hypertension due to hepatic cirrhosis is not solely due to vascular congestion and that other factors might also be important.

Radionuclide uptake/unit volume of spleen increases as liver disease gets worse (Table 3.5), signifying increasing blood flow with worsening liver disease. This is in agreement with the concept that splanchnic hyperaemia gets worse with worsening liver disease.

L/S ratio is a useful parameter which reflects the ratio of liver to spleen blood flow. As the results of the present investigation suggest a good correlation of this ratio to

Child's grade, it can be used to assess and follow-up patients with cirrhosis of the liver.

3.2.3.6 Conclusions

In conclusion, the present investigation has used non-invasive and safe means to assess liver function in hepatic cirrhosis and showed a significant correlation of grade of liver dysfunction as assessed by Child-Pugh's system with indices like L/S uptake ratio of radioisotope and spleen uptake of colloid/unit volume of the spleen. Splanchnic blood flow changes with worsening of liver function and increasing spleen size have also been assessed indirectly, by this method, as the uptake of colloid by the R-E cells is dependant upon rate of blood flow.

SECTION 4

DIABETES MELLITUS AND HEPATIC CIRRHOSIS

4.1 INTRODUCTION

4.1.1 Pathogenesis of glucose intolerance and diabetes mellitus in cirrhosis of the liver.

Impairment of glucose tolerance and liver disease frequently occur simultaneously (Megeysi et al, 1967; Petrides and DeFronzo, 1989). Over the past decades, it has been well documented that 60-80% of cirrhotics have glucose intolerance and that overt diabetes develops in up to 30% of these patients (Megeysi et al, 1967). The diabetes that develops is usually mild and non-insulin dependant (NIDDM). Most cirrhotics exhibit increased peripheral insulin levels and severe insulin resistance (Proietto et al, 1980; Cavallo-Perin et al, 1985; Taylor et al, 1985). The impairment of insulin action lies in the peripheral tissues (muscles) and results from a defect in insulin-mediated glycogen synthesis (Kruszynska et al, 1988; Petrides et al, 1991). In contrast, hepatic insulin sensitivity is well-maintained as evidenced by a normal suppressive effect of insulin on liver glucose output at least in the early stages (Petrides et al, 1991).

Conflicting data exists regarding the pattern of insulin secretion in liver disease. Stimulation of insulin secretion by arginine (Greco et al, 1979), tolbutamide (Pelkonen et al, 1981), intravenous glucose (Magnusson et al, 1987) and oral glucose ingestion (Proietto et al, 1984) has been reported to be decreased (Greco et al, 1979, Pelkonen et al, 1981, Magnusson et al, 1987), normal (Magnusson et al, 1987) or increased (Proietto et al, 1984). These data have been derived from peripheral plasma insulin assays (after it has undergone dilution and the effect of hepatic first pass metabolism) or from C-peptide concentrations (which are assumed

to be a better index of insulin secretion). Insulin secreted from the endocrine pancreas in cirrhosis undergoes hepatic first pass metabolism, portosystemic shunting through collateral vessels and the effect of dilution before it is measured at the peripheral sites. It is therefore, important to measure insulin secretion at its origin (portal vein) before it undergoes changes, which could modify it, qualitatively and quantitatively. Direct measurement and quantification of pattern of insulin secretion in man has not been done previously, although data from the dog model is available (Porksen et al, 1995b).

4.1.1.1 Methods of quantifying insulin secretion and action

The glucose clamp technique

A comprehensive understanding of the mechanism of disorders of glucose homeostasis in man requires the assessment of multiple physiological variables. Among the primary variables are the beta-cell response to glucose and the sensitivity of body tissues to insulin. There have been attempts to derive these variables from data obtained on glucose tolerance tests. In the oral version of the test, plasma glucose concentration rises and then falls; mean glucose curves derived from large populations are smooth, but curves on individuals are mainly erratic. Plasma insulin concentration curves generally follow a similar but lagging time course. In order to relate the response of the beta-cell (as assessed by changes in plasma insulin concentration) to the ever-changing stimulus of the plasma glucose concentration, the use of an "insulinogenic index" has been proposed and widely used (Perley et al, 1966; Seltzer et al, 1967). The index is computed from the ratio of the insulin-to-glucose (I/G) concentrations. The same data has been used (plasma glucose and

insulin values) to compute an index of tissue sensitivity to insulin, the glucose-to-insulin ratio (G/I ratio). The problem in using such a ratio results from the feedback loop relating these two variables and from the fact that neither variable is held constant. Thus, a rise in plasma glucose concentration stimulates the beta-cell release of insulin, the resultant rise in plasma insulin concentration stimulates the cellular uptake of glucose, and the plasma glucose concentration falls. These processes are, however, not sequential as described above, but occur simultaneously.

Studies of these two processes *invitro* are not subject to these problems. The excised pancreas or isolated islet preparation can be perfused at fixed hyperglycaemic levels and individual tissues can be perfused at fixed insulin concentrations. It is with this type of control in mind that the glucose clamp technique has been developed. The technique places the plasma glucose concentration under the investigator's control and thus breaks the simple glucose-insulin feedback loop. Both beta-cell sensitivity to glucose and tissue sensitivity to insulin can be quantified.

In the *hyperglycaemic clamp technique*, (DeFrenzo et al, 1979) the plasma glucose concentration can be raised and maintained at a chosen hyperglycaemic concentration within narrow limits. Because the stimulus to the beta-cell is controlled, studies of beta-cell sensitivity among different subject populations can be reliably compared. Furthermore, the maintenance of the same steady state plasma glucose concentration in all subjects obviates the need for using the I/G ratio and allows the plasma insulin response to be assessed directly. The hyperglycaemic

clamp has a major advantage in that, unlike the OGTT, the time course of the amount of glucose metabolized by the body can be quantified.

4.1.2 Diabetes mellitus and TIPSS insufficiency.

TIPSS is an effective method of treating portal hypertension and its complications (Jalan et al, 1995a). However, shunt insufficiency and occlusion are major limiting factors in the success of this form of treatment (Latimer et al, 1998). Primary shunt patency at six months, one year and two years has been reported as 71.2%, 58.2% and 21.4% respectively (Stanley et al, 1996). Rates of shunt insufficiency have however been shown to vary from 25-80% in different studies (LaBerge et al, 1993; Martin et al, 1993; Peramau et al, 1993; Jalan et al, 1994; Lind et al, 1994; Rossle et al, 1994). Shunt stenosis can be treated by balloon angioplasty, extension of the stent or the insertion of a co-axial stent, but re-occlusion occurs. (Dabos et al, 1996).

The pathogenesis of shunt stenosis is not entirely clear. One of the factors determining this is the time course of occlusion. Whilst early occlusion is associated with thrombosis, delayed shunt dysfunction is predominantly due to pseudointimal hyperplasia within the shunt (Henri et al, 1997). A number of factors have been assessed as predictors of shunt insufficiency (Jalan et al, 1995b) and biliary leak into the shunt lumen has also been implicated (Jalan et al, 1996a).

Diabetes mellitus, which is common in patients suffering from cirrhosis of liver, (Shearman and Finlayson, 1989) leads to smooth muscle cell proliferation and endothelial cell dysfunction in blood vessel walls. This is secondary to a number of growth factor abnormalities, which develop in this condition. (Aronson et al, 1996). These vascular changes could also be responsible for delayed TIPSS insufficiency due to pseudo-intimal hyperplasia within the stent lumen.

4.1.2.1 Histopathology of pseudo-intima

Pseudo-intima grows over a metal meshwork stent surface, over a period of weeks to months. The process starts off as a poorly organized fibrin and platelet clot mixed with inflammatory and red blood cells. The covering thickens with time as collagen fibres get more dense because the cellular component mainly consists of fibroblasts and myofibroblasts. After about two weeks, in all stents the fibrous layer covering the stent gets topped by a continuous layer of mature endothelial cells. The thickness of the pseudointima varies and in the intraparenchymal portion of the prosthesis ranged from 200-3,500 μm with maximal values most often recorded within the midst of the shunt (Ducoin et al, 1997).

4.2 STUDIES RELATING TO DIABETES MELLITUS AND CIRRHOSIS:

4.2.1 Measurement of pulsatile insulin secretion in patients suffering from hepatic cirrhosis.

4.2.1.1 Introduction

Insulin is secreted in high frequency pulses from the β cells of the pancreas into the portal vein (Goodner et al, 1977). This pattern of insulin secretion has been shown to be abnormal in subjects with Type 2 diabetes (Lang et al, 1981), their first-degree relatives (O'Rahilly et al, 1988) and patients at risk of developing Type 1 diabetes (Bingley et al, 1992). Diabetes mellitus that develops in hepatic cirrhosis resembles Type 2 diabetes in its pathogenesis and manifestations and in the later stages is accompanied by abnormalities of insulin secretion.

However, the quantification of pulsatile insulin secretion is complex. Insulin pulses are secreted into the portal circulation and undergo significant hepatic extraction and waveform attenuation before entering the systemic circulation (Porksen et al, 1995a). A canine model for direct portal vein catheterization has been developed to overcome this problem (Porksen et al, 1995b). Using this model, it has been reported that sampling from the systemic circulation results in an underestimate of both the frequency of insulin pulses and the calculated proportion of insulin secreted in the pulsatile mode (Porksen et al, 1995a). Studies of pulsatile secretion in humans have been confined to the systemic circulation (Lang et al, 1979; Hansen et al, 1982; Sonnenberg et al, 1992; O'Meara et al, 1993a; Porksen et al, 1997a, 1997b). These studies reported a pulse frequency of 15-20 minutes (Lang et al, 1981; O'Rahilly et

al, 1988; Bingley et al, 1992), but more recently a pulse frequency of about 6 minutes has been suggested (Porksen et al, 1997a, 1997b). It is speculated that these differences are due to the greater sensitivity of novel insulin assays applied in the later studies. If the true frequency of insulin pulses delivered into the portal circulation were 6-8 minutes, it would be comparable to the frequency observed in the isolated perfused pancreas (Stagner et al, 1985) as well as the perifused islets (Gilon et al, 1993; Marchetti et al, 1994).

4.2.1.2 Aims:

The present study was carried out using a previously validated deconvolution technique for quantifying pulsatile insulin secretion to plasma insulin concentration profiles measured simultaneously from the portal vein and the systemic circulation in human subjects with stable and compensated cirrhosis of the liver and *in situ* TIPSS .

The TIPSS catheter allowed relatively non-invasive high frequency sampling of blood from the portal vein in conscious human subjects (Stanley et al, 1996). The insulin secretion was quantified in the fasting basal state as well as during a hyperglycaemic clamp study.

Using this protocol, the present study has attempted to address the following questions. Firstly, is the frequency of pulsatile insulin secretion in man (as observed by direct sampling from the portal vein) comparable to the frequency reported previously from studies in isolated pancreatic islets and the isolated pancreas? Secondly, the frequency and amplitude of secretory bursts in the portal vein has been compared to that in the systemic circulation in order to see the impact of hepatic extraction and dilution on the peripheral delivery of insulin. Thirdly, does hyperglycaemia in humans (as in the dog model) enhance insulin secretion through augmentation of the mass of insulin bursts?

4.2.1.3 Patients and Methods

This study was approved by the Lothian ethics committee and all included subjects provided informed written consent.

Inclusion criteria included, patients with a patent TIPSS, stable compensated cirrhosis and normal OGTT. Exclusion criteria included decompensated liver disease, diabetes mellitus or impaired glucose tolerance, thrombosed stent and prolonged prothrombin time.

All patients underwent a 75 gms. OGTT 1-2 weeks prior to the study to exclude diabetes mellitus. Of the six patients screened, one had to be excluded because of diabetes. Patient details are presented in Tables 4.1 and 4.2.

Five patients with *in situ* TIPSS were studied immediately after a routine follow up portogram to check for TIPSS patency. The patients were admitted overnight to the Royal Infirmary of Edinburgh. After an overnight fast, a catheter inserted via the right jugular vein was guided under fluoroscopic cover through the TIPSS into the portal vein. The patency of the TIPSS was thus ensured. A sampling catheter was then inserted via the right internal jugular introducer sheath into the portal vein to allow sampling from the portal vein.

Table 4.1: Patient Characteristics in alcoholic cirrhosis.

Case	Age (years)	Sex	Weight (kg)	BMI (kg/m^2)	FPG (3.9 – 6.1) mmol/L
1.	58	F	60	23	4.4
2.	61	M	71	22	5
3.	43	M	71	24	4
4.	61	M	100	33	5.8
5.	54	M	117	36	5.4

FPG = fasting plasma glucose

M= male

F= female

BMI = body mass index

Kg= kilogram

Mmol/l= millimoles/l

Table 4.2: Liver function studies in alcoholic cirrhosis

Case no.	Alk Phos (40-125 u/L)	GGT (5-35 u/L)	Bilirubin (2-17 μM)	Albumin (36-47 g/L)	ALT (10-40 u/L)	INR (2-4.5)
1	125	124	56	32	14	1.4
2	80	20	17	43	27	1
3	146	100	42	34	56	1.3
4	135	61	147	35	35	1.7
5	92	54	17	41	33	1.2

ALT = alanine amino transferase

Alk Phos = alkaline phosphatase

GGT = gamma-glutamyl transferase

INR = International Normalized Ratio

4.2.1.4 Study protocol

All patients were admitted to the programmed investigation unit of the Royal Infirmary of Edinburgh the night prior to the study and remained fasting overnight. On the morning of the study, each subject underwent TIPSS portogram by a radiologist and the sampling catheter was placed in the portal vein at the end of this procedure.

A peripheral indwelling catheter for sampling purposes was also inserted in a dorsal hand vein and the hand was warmed to 40^0 C by an electric blanket to enable sampling of arterialized blood from this site. Although cirrhotic patients are already vasodilated warming of the hand to 40^0 C further vasodilates the hand circulation and makes the venous blood arterialized. An intravenous catheter was also placed in an antecubetal vein in the contralateral arm and infused with saline at a rate of 30 ml/hour.

Once all the catheters were in place, there was a 45 min rest period prior to the commencement of the protocol. At protocol time 0-40 min, simultaneous 1 min sampling of arterialized blood and portal vein blood was performed to obtain the insulin concentration profile each minute at each site in the fasting state. At protocol time 40 min, a hyperglycaemic clamp was commenced to raise the arterialized plasma glucose concentration to 8-9 mmol/l. This was achieved by infusion of a variable rate glucose infusion (Dextrose 50%) controlled by a programmable infusion pump (Harvard Infusion Pumps, Ayer, MA) connected to a personal computer.

Arterialized blood was sampled at 5 min intervals and the plasma glucose measured within 2 min. Steady state hyperglycaemia was achieved by protocol time 80 min (40 min after the clamp was begun) and the second intensive (1 min) sampling period began.

From protocol time 80-120 min, blood was sampled at 1 min intervals from the peripheral arterialzed line and the portal vein catheter simultaneously to determine the insulin concentration profile during the hyperglycaemic clamp. Portal vein sampling was completed in all five-study subjects in both the basal and hyperglycaemic sampling periods. Arterialized sampling was completed in four subjects only, as the peripheral sampling catheter was not working reliably.

In two subjects, portal vein blood flow volume (mls/min) was measured during the study (pre- and post-sampling), using doppler scanning.

4.2.1.5 Assays

Plasma glucose concentrations were measured by the glucose oxidase method using a Beckman glucose analyzer (Beckman, Palo, Alto, CA).

Plasma insulin concentrations were measured in duplicate by two-site immuno specific insulin ELISA (Andersen et al, 1993). In brief, the assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmmark) specific for human insulin. The detection range of this insulin ELISA was 5-600 pmol/l. At

medium (150 pmol/l), medium-high (200 pmol/l) and high (350 pmol/l) plasma insulin concentrations, the interassay coefficients were 3.7%, 4.0%, 4.5% and the corresponding intra-assay variations were 2.3%, 2.1%, 2.0%. There was no cross reactivity with proinsulin and split 32, 33 and des 31, 32 proinsulins respectively.

Plasma C-peptide concentration was assayed with a commercially available kit (K6218; DAKO, Cambridgeshire, UK). The assay is a two-site ELISA based on two monoclonal antibodies, using the same principles referred to above. Each sample was assayed in duplicate; intra- and interassay variation coefficients were 2.2% and 3.3% respectively.

4.2.1.6 Data analysis

Detection and quantification of pulsatile insulin secretion by deconvolution analysis

The plasma insulin concentration time series were analyzed by deconvolution as previously validated (Veldhius et al, 1995) to detect and quantify insulin secretory bursts (Porksen et al, 1995b). Deconvolution of plasma insulin concentration data was performed with a multiparameter technique that requires the following assumptions.

Plasma insulin concentrations measured in samples collected at 1 min intervals were assumed to result from 3 determinable and correlated parameters:

1) a finite number of discrete insulin secretory bursts occurring at either regular or randomly dispersed times and having:

a) individual amplitudes (maximal rate of secretion attained within a burst) and mass (integral of the calculated secretory event) and
b) a common half-duration (duration of an algebrically Gaussian secretory pulse at half-maximal amplitude), which are superimposed on
c) a basal time-invariant insulin secretory rate.

2) a bi-exponential insulin disappearance model in the systemic circulation, consisting of earlier directly estimated half lives of 2.8 and 5 min and a fractional slow component of 0.28 in healthy fasting humans;

3) a bi-exponential insulin disappearance model in the portal circulation, consisting of half lives of 1 and 3 min and a fractional slow component of 0.667. These parameters achieved the statistically best fit (maximally reduced fitted variance) of the portal-vein insulin concentration profile. All secretory rates were expressed as mass units of insulin (picomoles) released per unit distribution volume (litres) per unit time (min).

Statistical analysis

Data are presented as the mean ± s.e.m. Statistical comparison between groups was made by the Student's two-tailed t-test and a p value of <0.05 was taken as significant.

4.2.1.7 Results

The mean arterialized plasma glucose concentrations during the basal (fasting) and stimulated (hyperglycaemic clamp) sampling periods were 5.3± 0.3 and 8.1± 0.1 mmol/l respectively ($p<0.001$). As expected, there was a rise in the mean arterialized (basal vs. stimulated, 209±7.4 vs. 456±16.3 pmol/l, $p<0.001$) and mean portal vein (basal vs. stimulated, 440±25.3 vs. 1020.7±72.3 pmol/l, $p<0.001$) insulin concentration following the increase in the plasma glucose concentration from 5.3 to 8.0 mmol/l during the hyperglycaemic clamp.

The mean arterialized C-peptide concentration also increased with hyperglycaemia (basal vs. stimulated, 1.8±0.1 vs. 2.7±0.1 nmol/l, $p<0.001$) confirming an increase in insulin secretion in response to the glucose stimulus. Throughout the study in each subject, the portal-vein insulin concentration was higher than the corresponding arterialized insulin concentration in both basal ($p<0.001$) and stimulated ($p<0.001$) sampling periods (Figure 4.1).

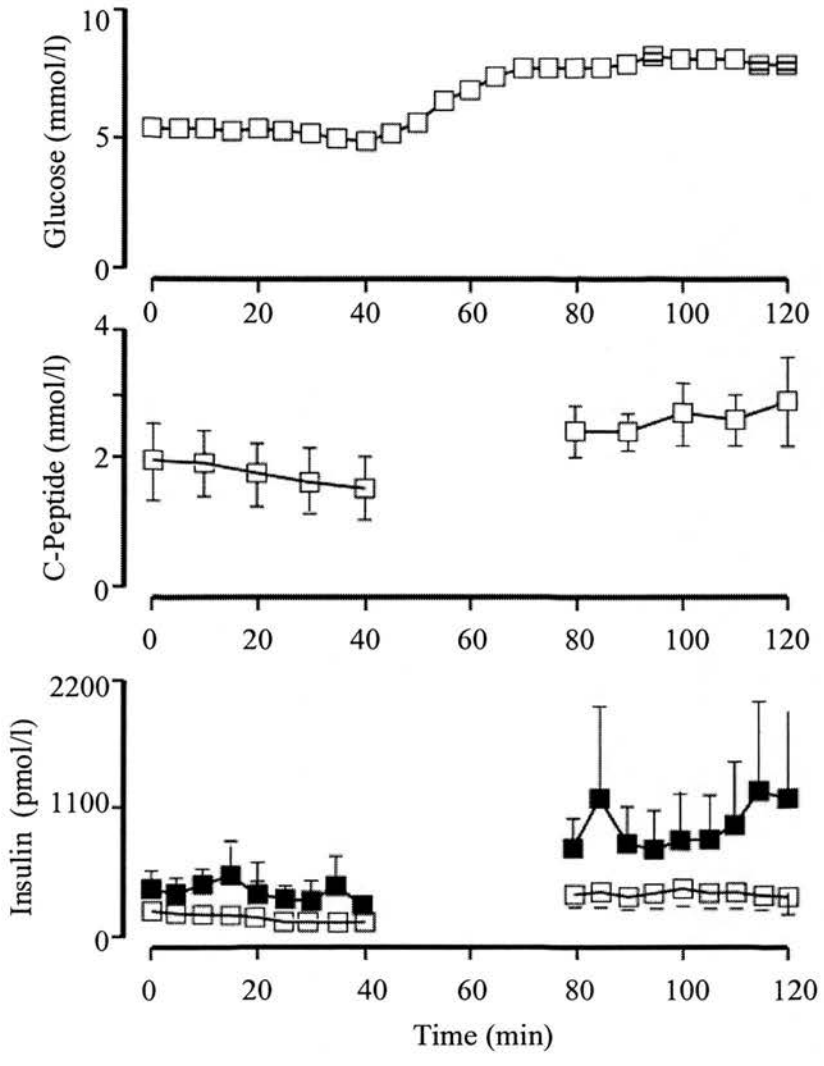

Figure 4.1: Mean arterialized plasma glucose (top panel) C-peptide (middle panel) and both arterialized (open square) and portal-vein (solid squares) insulin concentrations. T=0-40 min corresponds to the basal state, hyperglycemia was achieved by t=80 min, when the second intensive sampling was undertaken.

Portal vein blood flow

The mean portal vein blood flow was measured in two cases and was 1.1 l/min and 0.8 l/min. There was no change in the portal vein blood flow between the basal state and the hyperglycaemic clamp.

Insulin concentration profiles (Figure 4.2, 4.3)

Inspection of the plasma insulin concentration profiles from the individual patients indicated the presence of recurrent oscillations in both arterial and portal circulations in all patients. The magnitude of these oscillations was much larger in the portal circulation (Figure 4.2) in both the basal and stimulated states compared with the corresponding profiles from the systemic circulation. The amplitude of the oscillations increased during hyperglycaemia (Figure 4.2). The range of the insulin pulse amplitude observed in the portal circulation was 100-1000 pmol/l in the basal state and increased to 200-3000 pmol/l during the hyperglycaemic clamp. This contrasts with the corresponding range of pulse amplitudes observed in the systemic circulation of 10-30 pmol/l in the basal state and 40-100 pmol/l in the stimulated state (Figure 4.3).

Pulse detection

When the insulin concentration profiles were subjected to deconvolution, insulin pulses were invariably identified in both the portal and systemic circulation (Figure 4.4a & b, 4.5a & b). The pulse mass increased in response to hyperglycaemia in both portal (basal vs. stimulated, 418±155 vs. 1078±368 pmol/l, $p<0.05$) and systemic (basal vs. stimulated, 75±110 vs. 241±61 pmol/l, $p<0.05$) circulation (Figure 4.6).

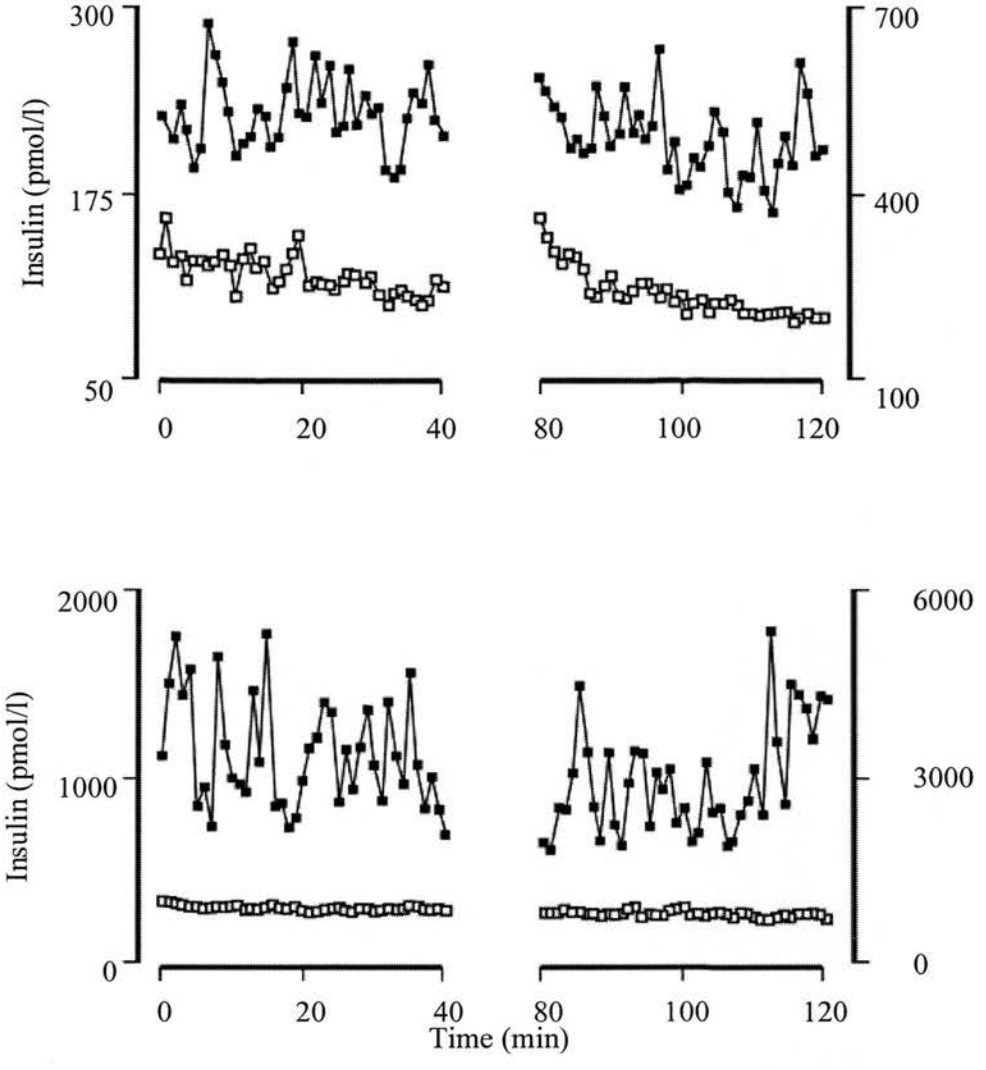

Figure 4.2. Plasma insulin concentration profiles from the basal (t=0-40 min) and hyperglycemic clamp (t=80-120 min) period in case 1 and 2 obtained from the arterialized (open squares) and portal-vein (solid squares) sampling sites. Note that the scales are adjusted in the left and right panels to accommodate the insulin concentration range observed. In all cases, oscillations in insulin concentrations are much greater in the portal-vein than in the arterialized sampling site.

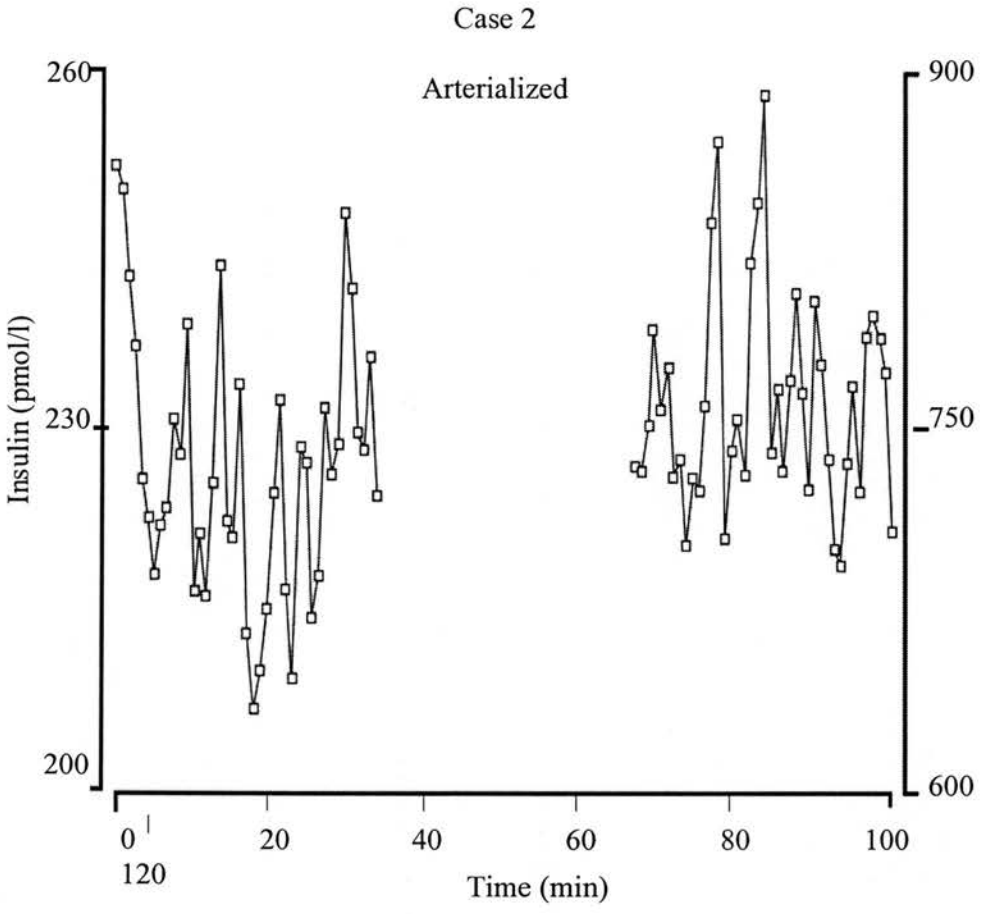

Figure 4.3: Plasma insulin concentration profile observed from the arterialized sampling catheter in case 2 in the basal state (t=0-40 min) and during hyperglycemia (t=80-120 min). Note that the scale has been adjusted for each sampling period to maximize the visualization of oscillations. In comparison with the arterialized insulin concentration profiles in Figure 4.2, the expanded scale clearly illustrates the prominent oscillations in insulin concentration in the both basal and stimulated state.

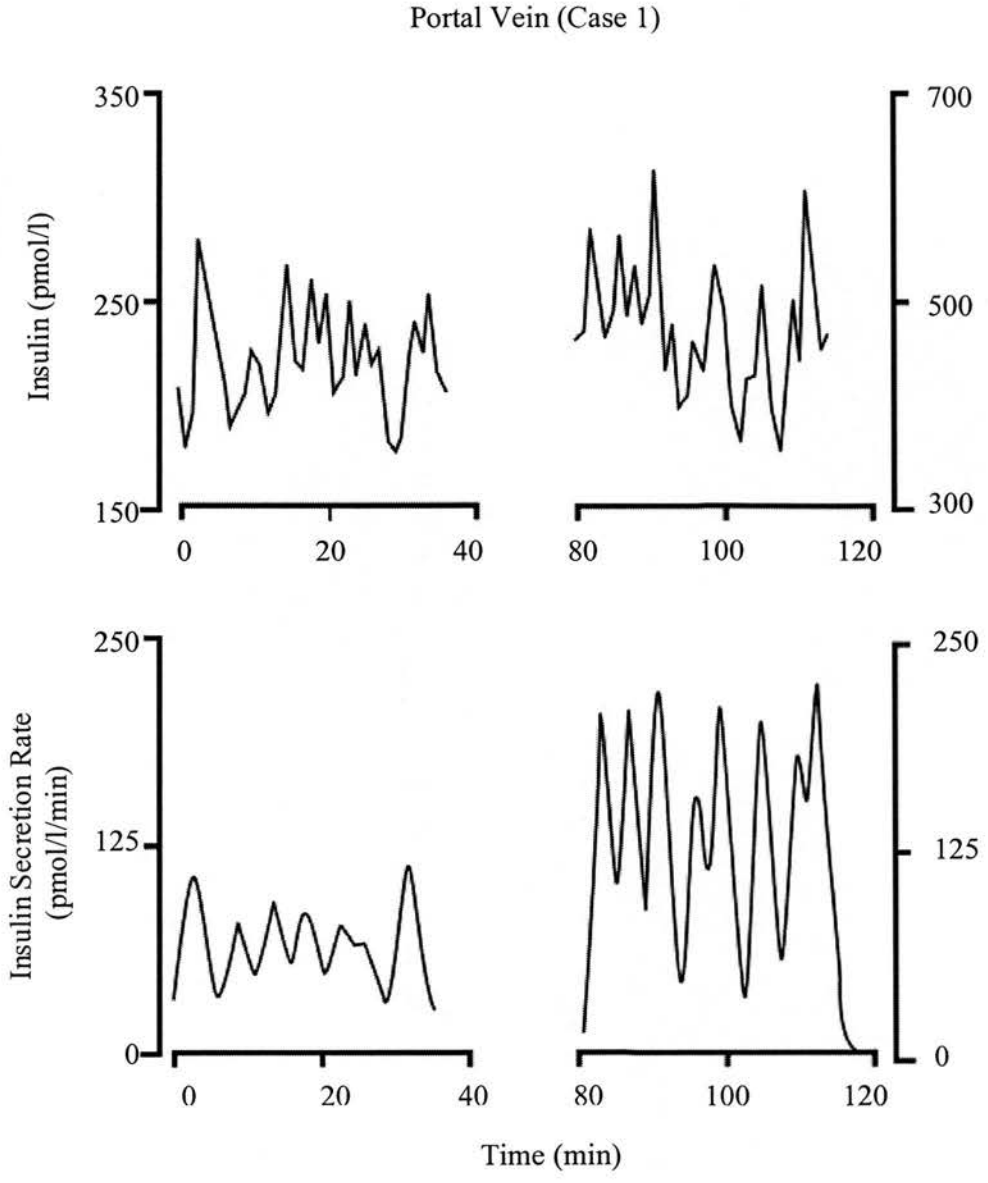

Figure 4.4a: The portal-vein insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal state (left panels) and during the hyperglycemic clamp periods (right panels) for case 1.

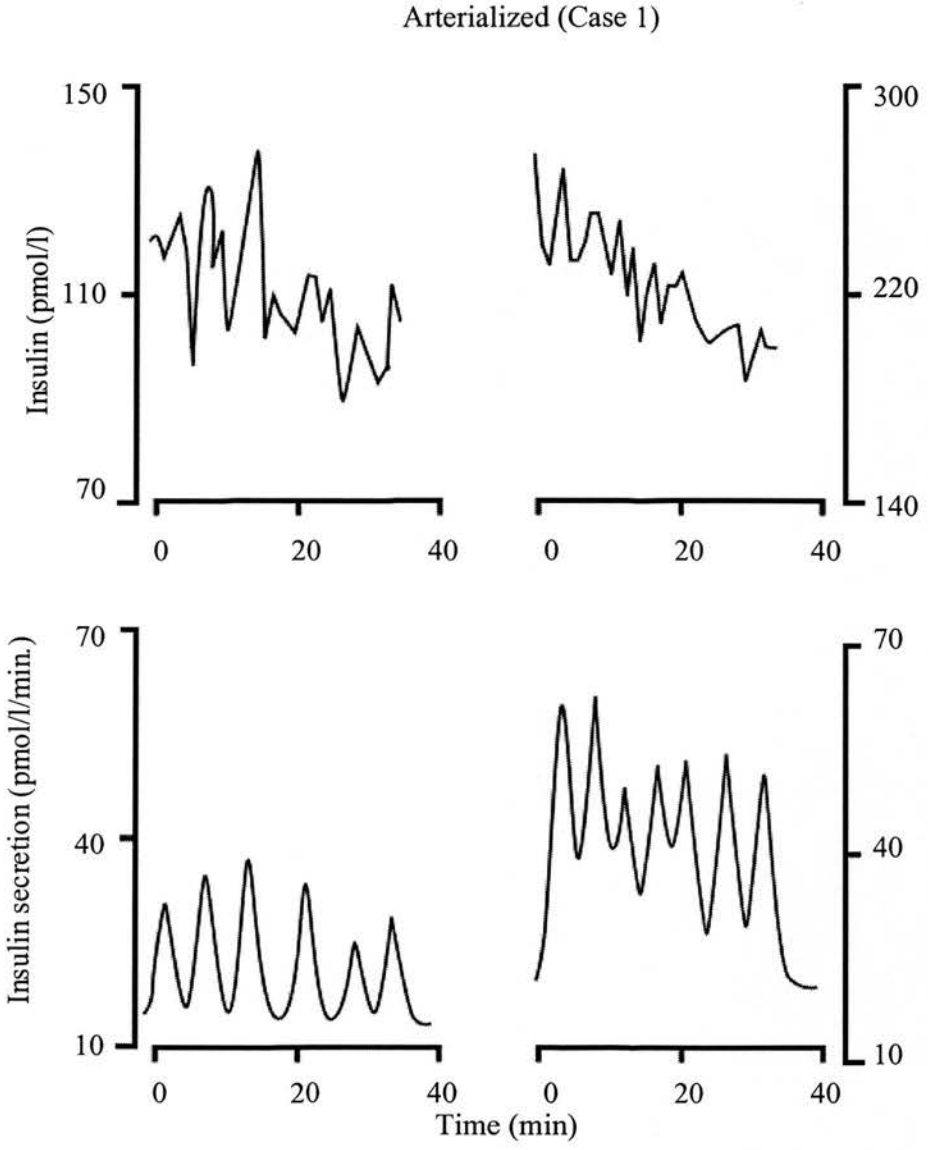

Figure 4.4b: The arterialized insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal state (left panels) and during the hyperglycemic clamp periods (right panels) for case 1.

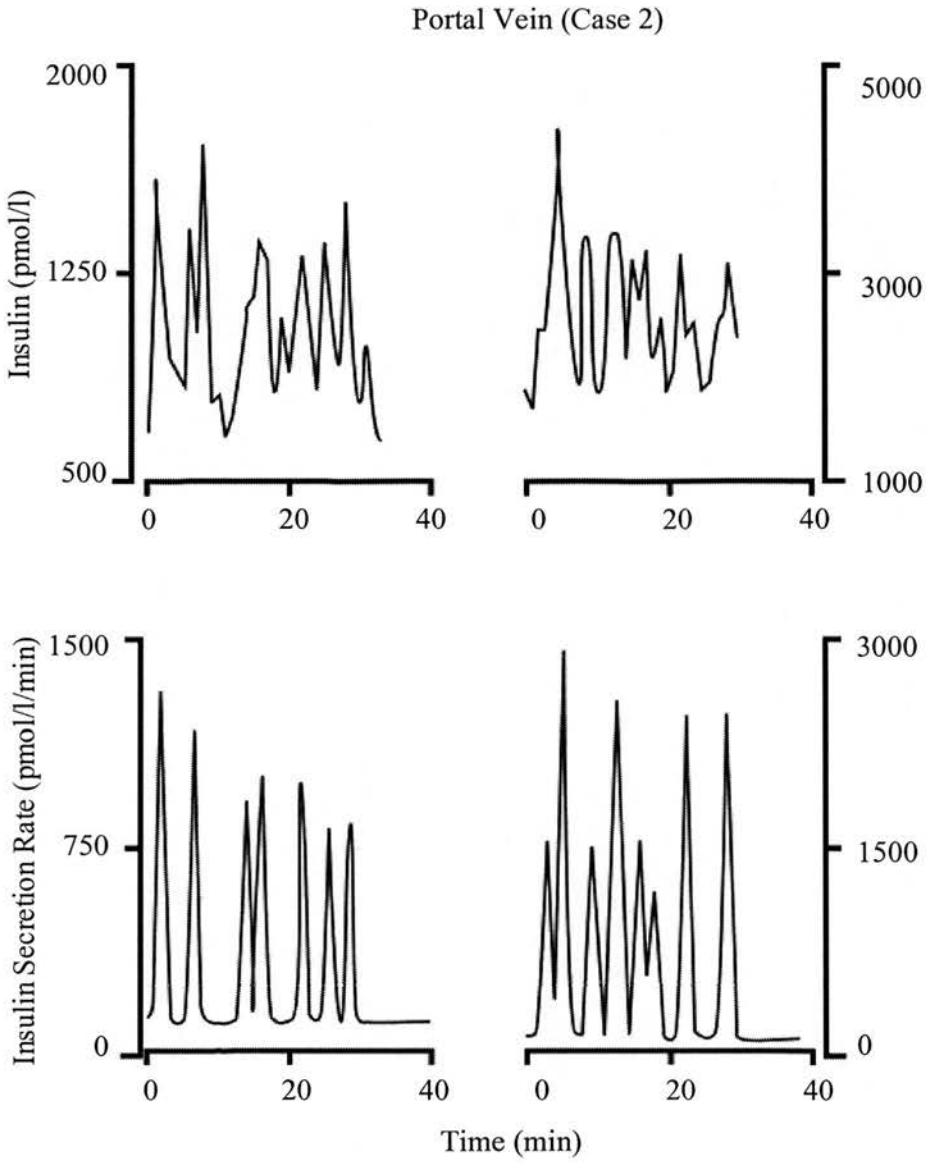

Figure 4.5a: The portal-vein concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal state (left panels) and during the hyperglycemic clamp periods (right panels) for case 2.

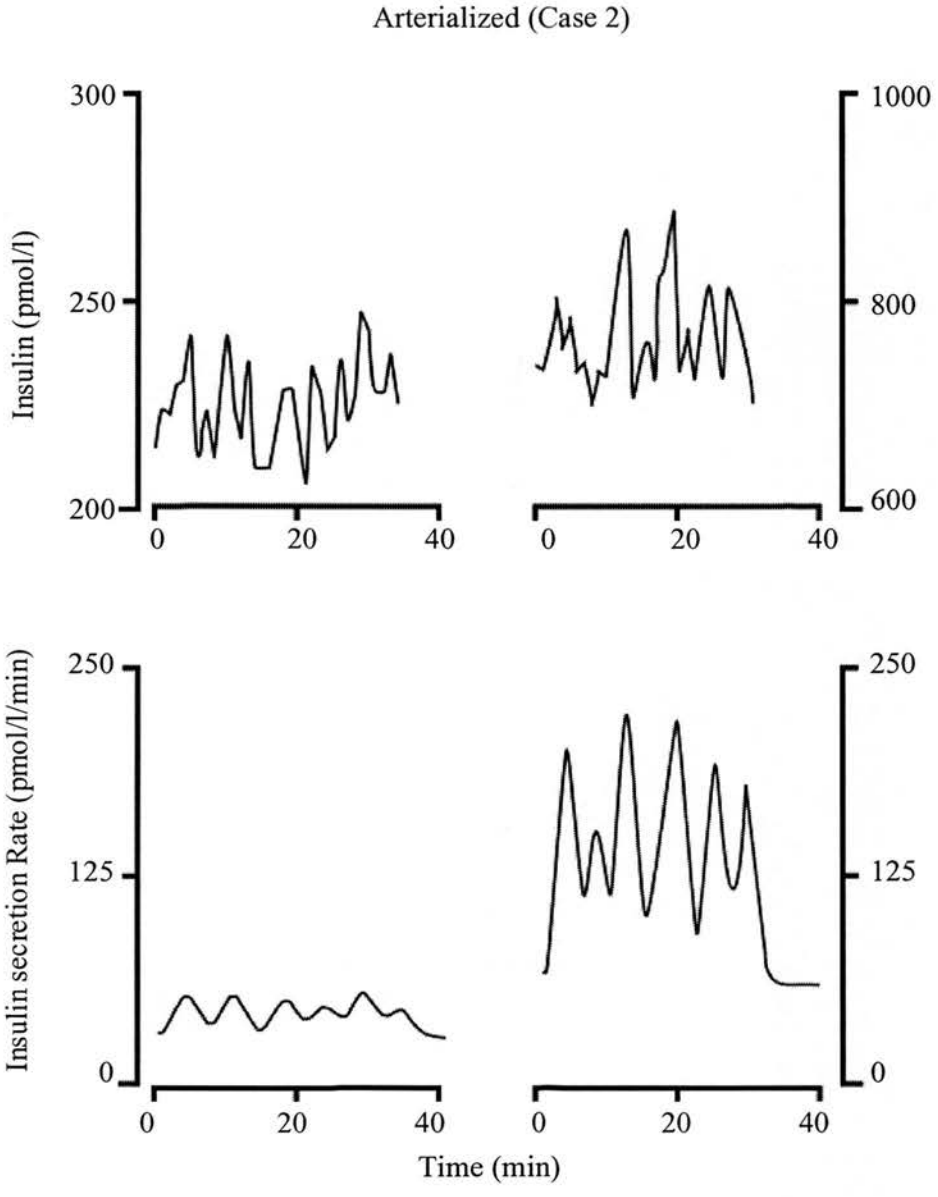

Figure 4.5b: The arterialized insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling period in the basal state (left panels) and during the hyperglycemic clamp periods (right panels) for case 2.

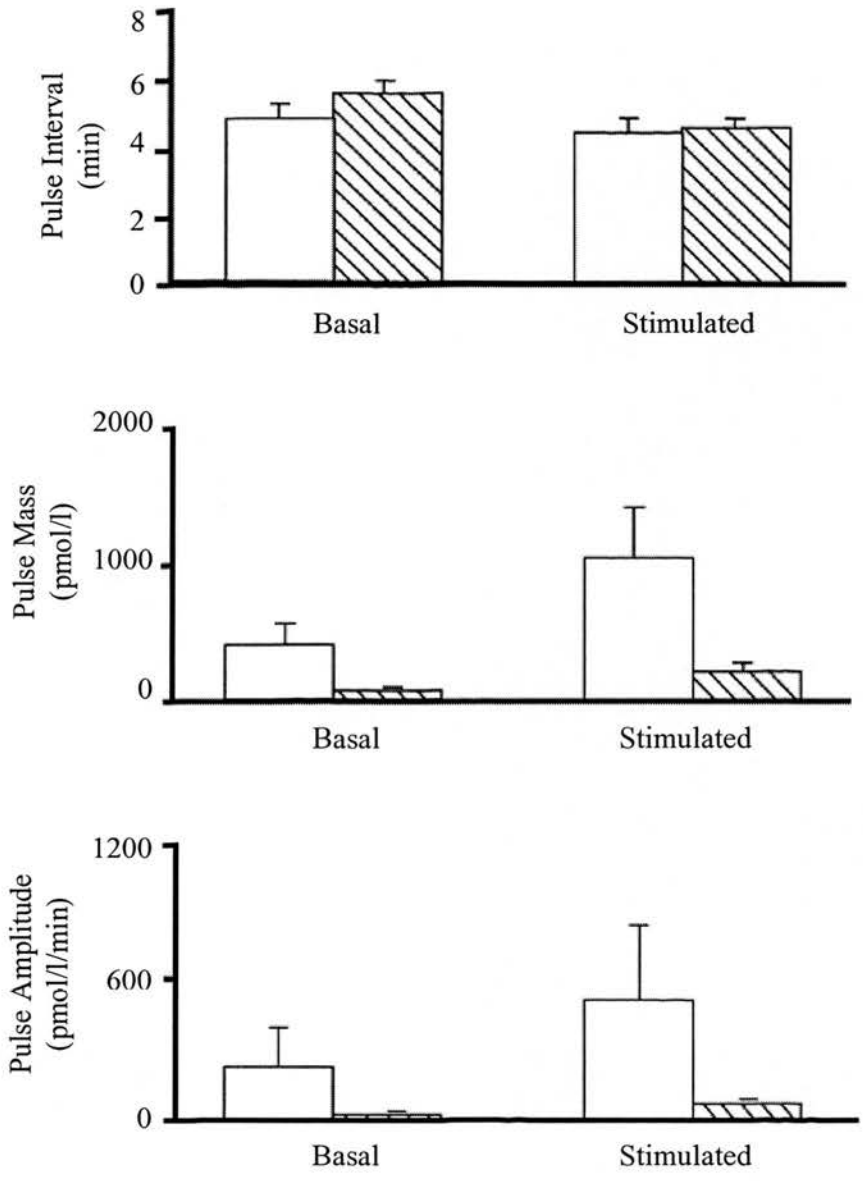

Figure 4.6: The mean insulin inter-pulse interval (top panel), pulse mass (middle panel) and pulse amplitude (bottom panel) during the basal sampling period and stimulated hyperglycemic period evaluated by sampling from the portal-vein (open panels) versus the arterial catheter (shaded panels). Units are minutes (pulse interval), pmol/l (mass), and pmol/l/min (amplitude).

Furthermore, the measured pulse mass/ volume of distribution was about 5 fold greater in the portal circulation in both the basal and stimulated states. The pulse amplitude determined by deconvolution was also markedly larger (7-8 fold) in the portal circulation in both basal (portal vs. systemic, 254±158 vs. 23±2.7 pmol/l/min) and stimulated (portal vs. systemic, 524±337 vs. 72±2.7 pmol/l/min) states confirming the impression gained by inspection of the insulin concentration profiles.

The inter-pulse interval (Figure 4.6) determined by direct portal-vein sampling was similar ($p>0.05$) in both the basal sampling period as well as during the clamp (Figure 4.6). The pulse interval detected by sampling from the systemic circulation was slightly but not significantly higher than that observed in the portal circulation.

4.2.1.8 Discussion

The present study uses direct and high frequency sampling from the portal vein to confirm that insulin is secreted in humans almost exclusively in discrete secretory bursts and that hyperglycaemia enhances insulin secretion by selective amplification of the mass of insulin contained within each secretary burst. Furthermore, we could establish that the prehepatic frequency of insulin pulses is about 5 min, which in fact corresponds with that observed in the portal vein of dogs *in vivo* (Porksen et al, 1995) and from the isolated perfused islets *invitro* (Gilon et al, 1993; Marchetti et al, 1994).

There has been some disagreement in the literature as to the frequency of pulsatile insulin secretion *in vivo* with the estimates ranging from once every 6 min. (Porksen et al, 1995a, 1995b) to once every 20 min. (Lang et al, 1979, 1981; O'Rahilly et al, 1988; Bingley et al, 1992; Sonnenberg et al, 1992). When pulsatile insulin secretion was measured directly from the portal vein in the dog, the interpulse interval averaged 6 min, based on conventional insulin immunoassay and a deconvolution analysis, which had been previously validated for insulin pulse detection (Porksen et al, 1995a, 1995b). Although the pulse frequency corresponded to that observed in perfused single islets *invitro*, it was three times greater than that observed previously in humans by sampling from the systemic circulation (Lang et al, 1979, 1981; O'Rahilly et al, 1988; Bingley et al, 1992; Sonnenberg et al, 1992). It was postulated that this large discrepancy was due to the relative loss of insulin pulses in the systemic circulation as compared to the portal circulation (Porksen et al, 1995b).

At least in the dog model, it has been confirmed that the sampling site is important when a conventional insulin assay is employed (Porksen et al, 1995a) since simultaneous estimates in the portal and systemic circulations revealed different interpulse intervals (Porksen et al, 1995a). The disadvantages of sampling from the systemic circulation were especially evident when the only available insulin assays were conventional immunoassays (O'Meara et al, 1993b). Even multiple replicates by these assays, which have cross reactivity with proinsulin and insulin split products, do not allow for fully reliable detection of high frequency insulin pulses in the systemic circulation. However, the introduction of the more sensitive and specific ELISA assays for insulin (Andersen et al, 1993) should theoretically overcome some of the problems. Recently, using an ELISA for insulin measurements, an insulin pulse frequency of ~ 8 min. has been reported when sampling from the systemic circulation in humans (Porksen et al, 1997a). The present study now confirms directly that the systemic sampling route can provide an accurate estimate of insulin pulse frequency *invivo* circulation in humans when the insulin concentration is measured by a sensitive and specific ELISA method and submitted to appropriate deconvolution based analysis.

Sampling directly from the portal circulation revealed a remarkable magnitude of insulin oscillations at this site. In the present study, the amplitude of insulin concentration in the portal vein was 100-1000 pmol/ l in the basal state and increased to 500-5000 pmol/l during hyperglycaemia. These values in the human are comparable to those observed in the canine portal vein (Porksen et al, 1995a, 1995b) and presumably reflect the insulin concentration wave front to which the liver is

exposed. The present study subjects were suffering from cirrhosis of the liver complicated by portal hypertension requiring treatment. Subjects with stable cirrhosis who were clinically well and had normal pre-study OGTT's were selected. However, portasystemic shunting of blood through TIPSS and liver damage due to cirrhosis of the liver may have resulted in less hepatic extraction of insulin. The present study presents a model, which can be used to study insulin secretion in cirrhotic patients in varying degrees of liver failure and glycaemic control.

Although some studies have reported that pulsatile insulin delivery to the peripheral circulation enhances insulin sensitivity, none has yet recapitulated the magnitude of insulin oscillations to which we observe the liver is exposed *invivo* during stimulation-enhanced insulin secretion (Matthews et al, 1983; Paolisso et al, 1990). The question remains, therefore, what the physiologic importance is, if any, of exposing the liver to such dramatic oscillations of insulin concentration. Since the pulsatility of peripheral insulin pulses is attenuated in patients with Type 2 diabetes (Laedtke et al, 2000), it is likely that the liver in these patients is exposed to even more attenuated oscillations in insulin pulse amplitude, which may contribute to the hepatic insulin resistance characteristic of this disease (De Fronzo et al, 1982).

The remarkable transhepatic attenuation of the insulin pulse signal from the portal circulation to the systemic circulation observed here is comparable to that observed previously in the dog (Porksen et al, 1995a). Although the attenuation may reflect up to 4-fold dilution of portal venous blood by the systemic venous drainage, the

insulin pulse amplitude is damped to a much greater extent than this (~ 10 folds). Presumably the latter reflects hepatic insulin clearance of insulin pulses.

4.2.1.9 Conclusions

In summary, direct sampling from the portal vein in humans establishes an insulin interpulse interval of ~ 5 min, and thereby resolves the discrepant insulin pulse frequency reported in cirrhotic humans with TIPSS insitu. A comparable insulin interpulse interval can also be detected by high frequency peripheral sampling with the use of highly sensitive assay and appropriately validated pulse detection. Direct portal vein sampling also reveals that in humans the majority of insulin is secreted in insulin bursts, and the apparently preferential hepatic extraction of pulses lead to post –hepatic delivery of insulin in smaller bursts. Finally, we confirm that the liver in humans is exposed to dramatic insulin concentration oscillations that far exceed the magnitude evaluated to date in clinical studies of insulin action *invivo.*

This study of insulin secretion, in human subjects has been carried out in patients suffering from cirrhosis of the liver and portal hypertension but it seems that liver damage and portasystemic shunting has not significantly influenced the peripheral delivery of insulin.

4.2.2 Role of Diabetes Mellitus in TIPSS insufficiency.

4.2.2.1 Background

TIPSS is an effective method of treating portal hypertension and its complications (Jalan et al, 1995a). However, shunt insufficiency and occlusion are major limiting factors in the success of this form of treatment (Latimer et al, 1998). Primary shunt patency at six months, one year and two years has been reported as 71.2%, 58.2% and 21.4% respectively (Stanley et al, 1996). (Please see section 4.1.2 and 4.1.2.1 for further details).

4.2.2.2 Aim

To assess the role of diabetes mellitus in producing TIPSS insufficiency.

4.2.2.3 Patients and Methods

The cohort of patients (n=248) who underwent TIPSS insertion between July 1991 and July 1997 was reviewed, looking for shunt dysfunction post-TIPSS and its relation to diabetes mellitus.

Patients were included in the analysis if TIPSS insertion was successful, at least one portographic shunt assessment was completed post-insertion, blood sugar profile was available for a diagnosis of diabetes mellitus to be made and early TIPSS insufficiency (within 1 month post-TIPSS) was absent. Patients were not entered into the analysis if they had incomplete data, portographic follow-up was not available, and early TIPSS insufficiency or HCC was present.

One hundred and seventy seven patients (71.4%) had successful TIPSS placement and had blood sugar profiles available for a diagnosis of diabetes mellitus to be made or excluded. Fifty-nine patients were excluded because of either early death, early shunt insufficiency, or referral for porto-systemic shunt surgery or liver transplantation within the first month following TIPSS insertion. The clinical data of the 118 patients who were entered into the final analysis comparing diabetic vs non-diabetic patients are presented in Table 4.1. TIPSS insertion was undertaken according to the method described elsewhere (Jalan et al, 1994). The procedure was carried out under sedation and analgesia with local anaesthesia, unless general

anaesthesia was indicated because of the clinical condition of the patient. Patients were followed up by doppler ultrasound at day seven post shunt placement to exclude early thrombosis. Regular follow up by portography was undertaken at six monthly intervals unless shunt occlusion was clinically suspected, which led to earlier portographic shunt assessment.

Data were censored when the patient died, had documented shunt insufficiency, had liver transplantation or portographic surveillance was discontinued.

Appendix VII shows gross morphology of an occluded shunt opened up after OLT. Histology of pseudointimal hyperplasia within an occluded TIPSS is shown in appendix VIII. Radiological appearance of pseudointimal hyperplasia as seen at check portography is shown in appendix IX,X and XI.

Definitions

PSI was defined as the increase in PPG to >12 mm Hg (Jalan et al, 1997). However, TIPSS is often inserted for the treatment of ascites, where the PPG may be <12 mm Hg at the time of stent placement. In such situations, SI was defined as an increase in PPG of 20% or more over the immediate post-TIPSS PPG (Jalan et al, 1994; Stanley et al, 1996).

PSI was further classified into early (<1 month post shunt placement) and delayed (>1 month post shunt placement). Diabetes was either diagnosed on OGTT or on the basis of two random blood glucose levels >11 mmol/l taken six months apart

(W.H.O. criteria). Rebleeding was defined as overt haematemesis and/or melaena or a drop of haemoglobin of greater than 2 g/dl without any other demonstrable source of blood loss or haemolysis. Endoscopy confirmed gastroesophageal variceal bleed or portal hypertensive gastropathy.

Statistical Analysis

The statistical analysis was carried out using the Statistical Package for Social Scientists (SPSS) software. In order to compare risk factors for shunt insufficiency in the diabetic and non-diabetic patients, univariate testing was first carried out. T-tests were used to compare mean age, pre-shunt PPG and post-shunt PPG, in the two groups of patients. Non-parametric Mann-Whitney tests were performed to compare length of follow-up and shunt-diameter in the two groups, and chi-square tests were performed to test for associations between presence of diabetes, gender, Child's class, primary delayed insufficiency and variceal haemorrhage.

In order to determine which factors were independently associated with primary delayed insufficiency, logistic regression modeling was carried out. Several clinical and biological variables were considered for inclusion in the model including age, gender, Child's class, shunt diameter, pre and post shunt PPG and whether the patient had diabetes. Kaplan-Meier survival analysis was used to compare time to delayed shunt insufficiency and a log rank test was applied to check for statistical significance of the difference.

4.2.2.4 Results

Diabetes was present in 28 out of 177 (15.8%) patients who had both a successful TIPSS and blood sugar profiles. Of the 118 patients who met inclusion criteria, 23 (19.5%) patients were diabetic and 95 (80.5%) non-diabetic. Statistical testing provided no evidence that the diabetic and non-diabetic patients differed in age, sex, pre-shunt PPG, post-shunt PPG and length of follow up (Table 4.3). However, the presence of diabetes was found to be associated with a better Child's class ($p=0.01$); diabetic patients were more likely to have a Child's class A or B (87.0%) than non-diabetics (56.8%). The median inserted shunt diameters were equal in the two groups, but the inter-quartile ranges showed diabetics to have larger shunts in place ($p=0.02$).

A comparison of clinical and demographic features between the study sample and the entire TIPSS treated population is presented in Table 4.7 and confirms the true representative nature of the sample in comparison to the TIPSS patient population from which it is derived.

The frequency of delayed PSI in diabetic versus non-diabetic patients is presented in Table 4.4. Diabetic patients were more likely to suffer from primary delayed insufficiency (87.0%) than non-diabetic patients (60.0%). This is unlikely to be due to a difference in Child's classes amongst the two groups as the incidence of shunt insufficiency is similar in Child's classes A & B compared with C (Table 4.5). There was no evidence of an association between the presence of diabetes and variceal haemorrhage post shunt insertion. Cumulative shunt patency is presented in Figure

4.7, which shows significantly reduced patency rates over time in diabetic patents with cirrhosis of the liver (p=0.04).

Table 4.6 presents odds ratios (ORs) and 95% confidence intervals (CIs) for the factors included in the final logistic regression model. These factors are statistically significant when included in the model, and are independently associated with delayed shunt insufficiency. Females are more likely than males, and diabetics are more likely than non-diabetics, to experience shunt insufficiency (OR = 3.44, 95% C.I. = 1.37 to 8.61, OR = 7.30, 95% C.I. = 1.87 to 28.43 respectively). However, individuals aged 55 years or over are less likely than those in the younger age group to experience shunt insufficiency (OR = 0.40, 95% C.I. = 0.17 to 0.94).

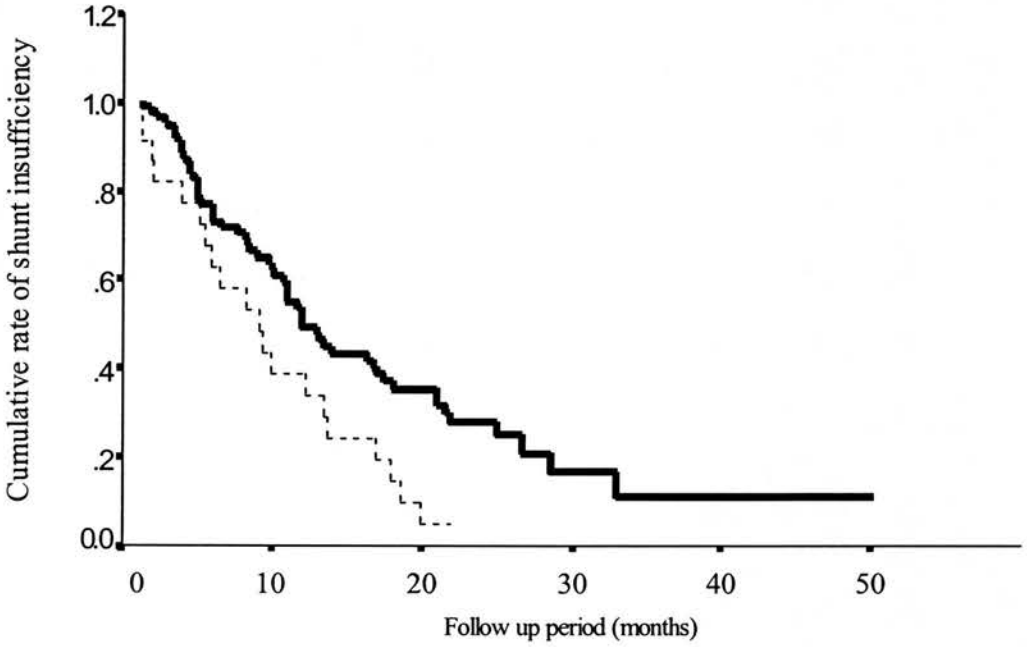

Figure 4.7: Cumulative rate of primary delayed shunt insufficiency compared in diabetic (dotted line) versus non-diabetic (solid line) cirrhotics p=0.04.

4.2.2.5 Discussion

This study included a large cohort of patients with TIPSS who underwent long-term portographic follow-up. Diabetes has been shown to be associated with increased prevalence of stent occlusion following coronary balloon angioplasty and stent implantation (Galan et al, 1986; Reinsing et al, 1993; Weintraub, 1993). If restenosis is viewed as an intraluminal growth process after vascular injury, similar mechanism might be operative in TIPSS occlusion as in coronary stent occlusion. Although there are haemodynamic differences between the high-pressure arterial circulation in the heart and the low-pressure portal venous circulation, the turbulent flow in the TIPSS, especially at the hepatic venous end of the stent makes it liable to injury from stress of the jet stream of blood. This is followed by the process of repair and endothelial regeneration and under certain circumstances may result in pseudointimal hyperplasia and shunt insufficiency (also see section 4.1.2.1).

In our study population, the diabetic and non-diabetic groups were comparable in their clinical characteristics except for Child's class and stent diameter. Child's class was better and stents used larger in the diabetic group. It is a bit surprising that the overall prevalence of diabetes was relatively low in Child's 'C' patients studied but this may be a reflection of low numbers in the cohort. Child's class, however, was not associated with increased prevalence of delayed PSI (Table 4.5) and larger diameter stents in diabetic cirrhotics should have made them less liable to occlude. Furthermore, all factors in Table 4.3 were considered for inclusion in the logistic regression model and Child's class and shunt diameter were not independent risk factors.

Table 4.7 very clearly demonstrates that although the study sample is only 118 (47%) of the entire TIPSS treated group, it is still comparable to and representative of the latter in its clinical and demographic features. The number of patients who underwent placement of TIPSS as an emergency and those with Child's grades A&B were proportionately slightly greater in the study sample as compared to that in the entire TIPSS treated population, but that did not achieve statistical significance ($p>0.05$). Although different complications of portal hypertension from liver diseases of varied aetiology led to treatment with TIPSS, the most commonly treated entity at the Royal Infirmary, where this research was carried out is oesophageal varices due to alcoholic cirrhosis.

The lack of evidence of an association between variceal haemorrhage post shunt occlusion and the presence of diabetes is surprising but may be due to the close follow up in our patients, which allowed early intervention. Some of the patients also underwent endoscopic band ligation after TIPSS, which would reduce the risk of bleeding.

On post-TIPSS follow-up, hepatic encephalopathy developed for the first time in 4/23 (17.4%) diabetic cirrhotics (Table 4.4) and needed standard therapeutic measures to be instituted. Although this was a higher incidence than in the non-diabetic patients, it was not statistically significant. The precipitation of encephalopathy did not follow episodes of gastro-intestinal bleeding and 2 patients each had Child's grades B & C respectively.

This is the first study looking at the role of diabetes mellitus in occlusion and insufficiency of TIPSS. The results of the present investigation indicate that the presence of diabetes is associated with delayed shunt insufficiency, independently of age and gender. Further work needs to be carried out to determine if this may be a possible causal effect. Hyperglycaemia induces alterations of growth factor secretion (Aronson et al, 1996), which may be important in the genesis of shunt narrowing due to fibrous tissue and endothelial overgrowth. The role of factors such as Insulin growth factor-1 (IGF-1), Tumour growth factor-beta (TGF-beta) and endothelins (which have increased expression and secretory rates in individuals with diabetes and hyperglycaemia (Taqkahashi et al, 1990; Meyer-Schwickerath et al, 1993; Yamamoto et al, 1993)) in smooth muscle cell replication and pseudo-intimal hyperplasia in the shunt lumen should be investigated.

4.2.2.6 Conclusion

From a practical standpoint, the results of this study would suggest that patients should be screened for diabetes at shunt implantation and a meticulous glycaemic control maintained post TIPSS to try to prevent hyperglycaemia, and therefore hopefully, minimize delayed shunt insufficiency.

Table 4.3. Comparison of clinical data and shunt characteristics in diabetic vs. non-diabetic patients undergoing TIPSS with no demonstrable early SI.

Factor	Diabetic (n=23)	Non-Diabetic (n=95)	p value
Age in years (mean ± SD)	57.3 ± 11.2	52.2 ± 12.5	0.07
Gender (Males/Females)	18/5	57 / 38	0.15
Child's Class (A+B/C)	20/3	54 / 41	0.01
Follow up in months (median; IQR)	15.0, 22.0	17.2, 18.2	0.43
Pre- shunt PPG mmHg (mean ± SD)	19.6 ± 5.5	19.2 ± 7.0	0.79
Post-shunt PPG mmHg (mean ± SD)	8.5 ± 4.0	6.8 ± 4.6	0.11
Stent Diameter in mm (median; lower Quartile, upper quartile)	12.0; 12.0,14.0	12.0; 10.0,12.0	0.02
Stent Diameter in mm (mean±s.e.m)	12.2 ± 0.32	11.44 ± 0.16	0.04

SD=Standard Deviation

IQR = Interquartile Range

s.e.m = standard error of mean

Table 4.4: Outcomes in diabetes vs. non-diabetic patients followed up for Primary Delayed Insufficiency.

Factor	Diabetic (n=23)	Non-Diabetic (n=95)	p value
Primary Delayed Insufficiency	20 (87.0%)	57 (60.0%)	0.02
Variceal Haemorrhage	4 (17.4%)	12 (12.9%)	0.52
Post-TIPSS Hepatic Encephalopathy	4 (17.4%)	12 (12.9%)	0.52

Table 4.5: Primary delayed shunt insufficiency and variceal haemorrhage in relation to Child's class of liver disease.

Outcome	Child's Class A+B (n=71)	Child's Class C (n=47)	p value
Primary Delayed insufficiency	52 (70.3%)	26 (56.8%)	0.16
Variceal Haemorrhage	11 (15.1%)	5 (11.6%)	0.78

Table 4.6: Final logistic regression model of primary delayed shunt insufficiency.

Factor	Number of Subjects	Odds ratio (95% Confidence Interval)	p value
Diabetes:			
Non Diabetic	95	1.00	<0.01
Diabetic	23	7.30 (1.87 - 28.43)	
Gender:			
Male	75	1.00	<0.01
Female	43	3.44 (1.37 - 8.61)	
Age:			
Less than 55 years	61	1.00	0.04
Greater than 55 years	57	0.40 (0.17 - 0.94)	

Table 4.7: Comparison of clinical data and shunt characteristics of the study sample vs. the whole group of TIPSS treated patients.

Factor	Study sample (n=118)	Total TIPSS treated patients (n=248)*
Age in years		
(mean ± SD)	53.4±12.7	53.7±12.9
Gender		
Male	73	156
Female	45	92
Indication for TIPSS		
Oesophageal varices	75	155
Gastric varices	19	40
Ascites	16	36
PHG	3	6
Oesophageal+Rectal varices	3	8
Hydrothorax	2	3
Aetiology of liver disease		
Alcoholic cirrhosis	81	161
Cryptogenic cirrhosis	5	21
PBC	10	19
Post hepatitic cirrhosis	12	20
Congenital fibrosis	3	6
PSC	3	5
CAH	3	6
Miscellaneous	1	10
Procedure		
Elective	45	141
Emergency	73	107
Child-Pugh Grades		
A+B	65	115
C	53	133

* Independent samples t-test and chi-square test was applied to test for association between the two groups and p value was found to be not significant in comparing different factors ($p>0.05$).

SD=standard deviation

PHG= Portal Hypertensive Gastropathy

PBC= Primary Biliary Cirrhosis

PSC= Primary Sclerosing Cholangitis

CAH= Chronic Active Hepatitis

EPILOGUE

The thesis has investigated the role of iNOS in modulating α-adrenoreceptor mediated vasoconstriction in the normal as well as cirrhotic hepatic arteries. There was no difference in the strength and sensitivity of contraction of the hepatic arteries obtained from cirrhotic compared to normal individuals, studied *exvivo*. This could partly be explained on the basis of the difference in time duration, which the donor (normal) and recipient (cirrhotic) blood vessels remained *exvivo* following transplant surgery and also the difference in storage media used for the two types of blood vessels. However, this should not be a significant factor affecting contractility. These experiments have therefore demonstrated clearly that neither α-adrenoceptor mediated (PE) nor α-adrenoceptor independent (KCl) vasoconstriction were impaired in hepatic arteries isolated from patients with advanced cirrhosis. This indicates that impaired excitation-contraction coupling in the muscle cells of hepatic arteries in cirrhotic individuals is not responsible for the impaired pressor response associated with this condition, *invivo*. Furthermore, inhibition of iNOS by incubation of the blood vessels with L-NNA made no significant difference to the contractility of the vessels indicating that the release of vasodilators from the vascular smooth muscle cell in arteries denuded of endothelium is not responsible for the impaired pressor response associated with this condition. Our results, therefore imply that an endothelium dependent factor is the important mediator of impaired vascular tone in cirrhosis.

Portal hypertension due to hepatic cirrhosis is also associated with a number of complications such as splenomegaly, hepatic encephalopathy, development of GOV and bleeding from them, ascites and metabolic complications like diabetes mellitus.

Splenomegaly is an important early sign of portal hypertension due to hepatic cirrhosis. In cirrhosis of the liver it signifies not only the presence of portal hypertension, but it is also associated with potentially important sequelae like hypersplenism. Our results have shown a definite correlation between spleen size, the size of GOV and bleeding from varices. This might be useful, clinically in monitoring the progression of disease.

We have studied two methods of measuring spleen size (ultrasonography and radionuclide). We also compared the spleen size as judged by these methods with actual spleen size as measured at autopsy. We have found that the spleen size measured by these two methods correlates well and also correlates well with direct measurement of spleen weight. This is an important observation in that it shows that a non-invasive method like ultrasonography can be used to assess spleen size accurately and indirectly monitor the size of GOV with disease progression.

Radionuclides in diagnostic imaging in hepato-biliary disease has largely been replaced by ultrasonography, MRI and CT scanning. However, the last two are expensive, not readily available and CT scanning exposes the patient to a large dose of radiation. Radionuclide uptake studies from our experiments have not only given useful information about the structure of the spleen but also help quantitate liver

function. A good correlation of spleen uptake of colloid/unit volume and L/S uptake ratio of colloid with liver function has been observed. Accordingly, radionuclides can still be used in clinical practice, where available, to assess liver function in chronic liver disease.

Another frequently observed and important complication of hepatic cirrhosis is diabetes mellitus. The pattern of insulin secretion in the portal vein of cirrhotic patients has been studied using TIPSS inserted patients as a model. This has not been studied before in human subjects and could be used in different categories of chronic liver disease to study the pattern of insulin secretion and its relation to the development of diabetes.

The impact of diabetes mellitus on TIPSS insufficiency has been studied. Diabetes mellitus has been found to be a significant independent factor, in the production of pseudointimal hyperplasia in TIPSS. This has practical implications in that Diabetes mellitus should be sought early in patients undergoing TIPSS and treated to try to reduce the risk of TIPSS occlusion.

In conclusion, this work has shed light on several aspects of cirrhosis and portal hypertension and helped elucidate many facets of complications of the disease, which may have clinical significance. This should help in understanding better, the clinical management of patients with portal hypertension due to hepatic cirrhosis.

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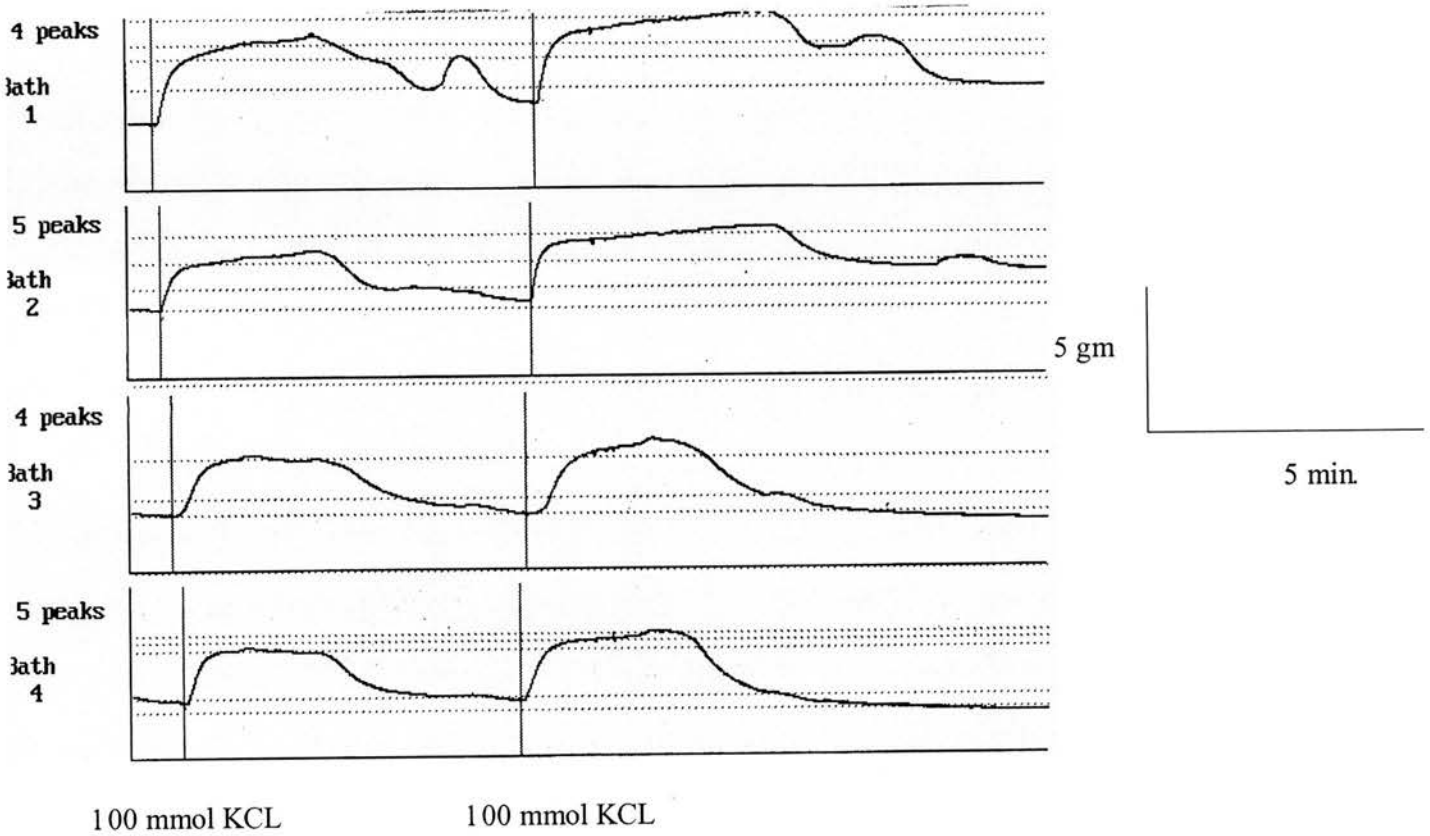
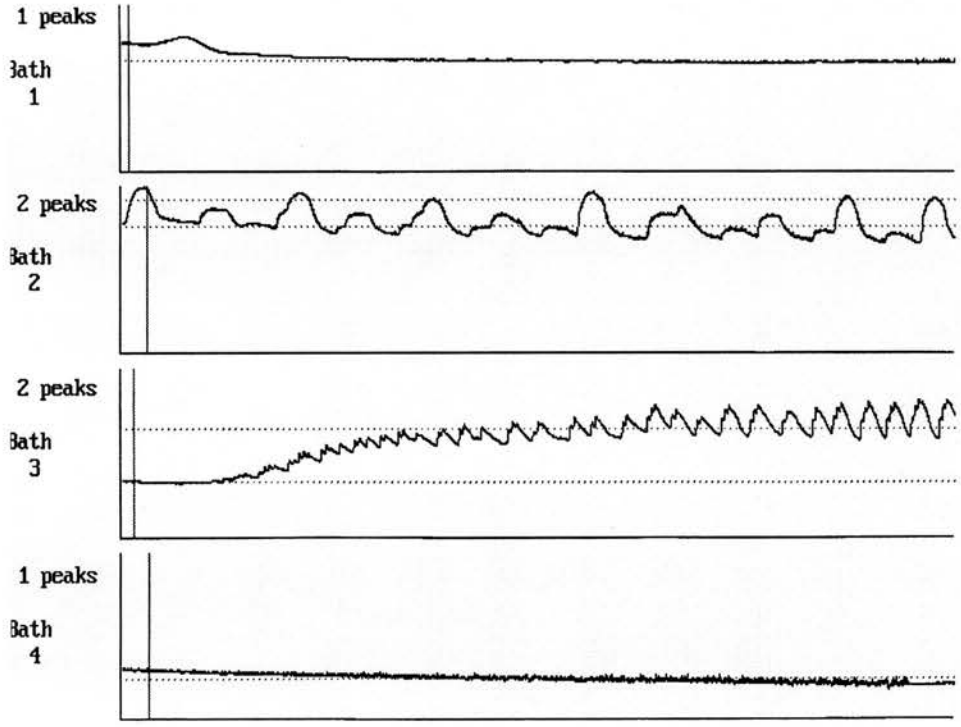
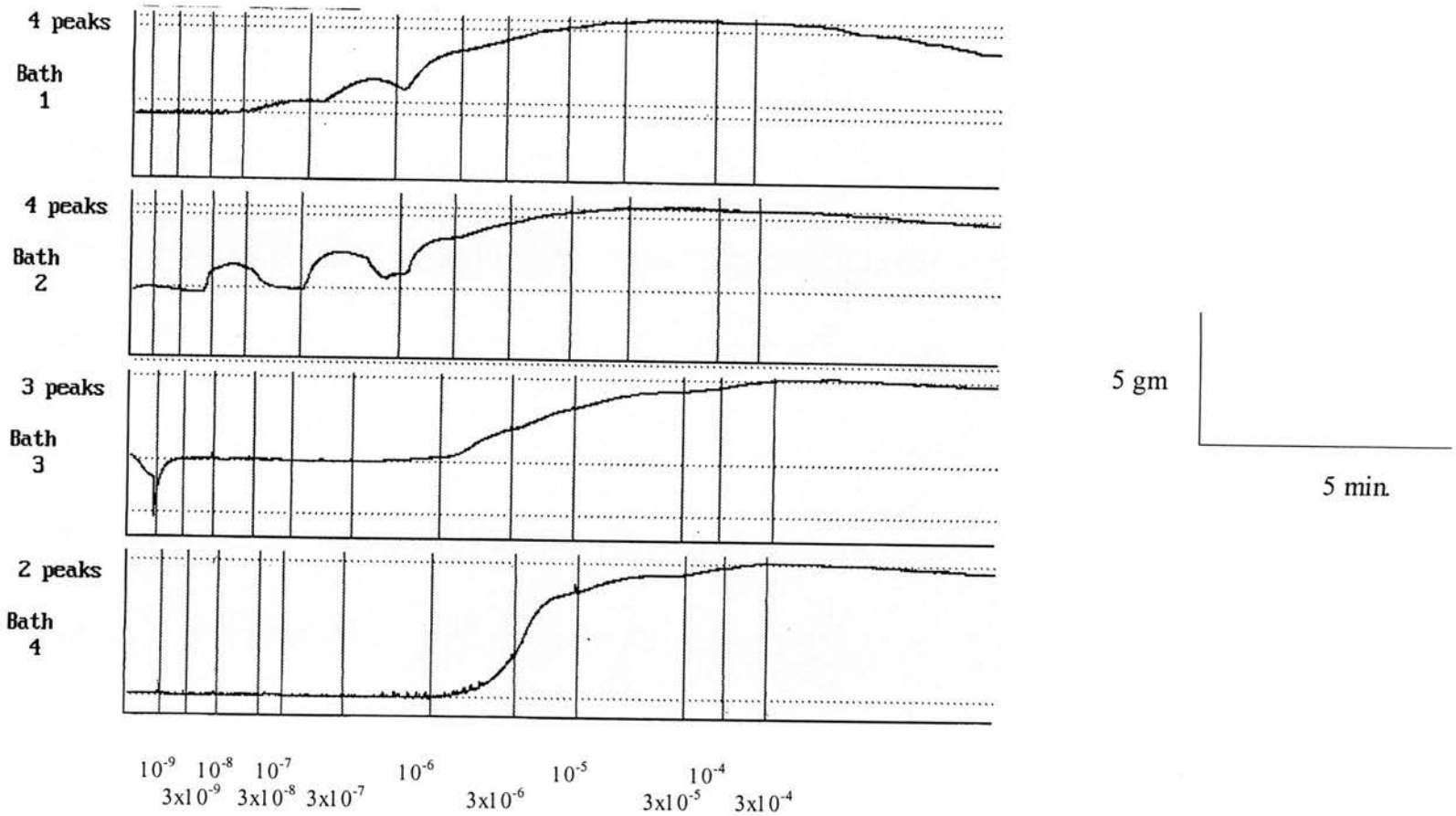
APPENDICES



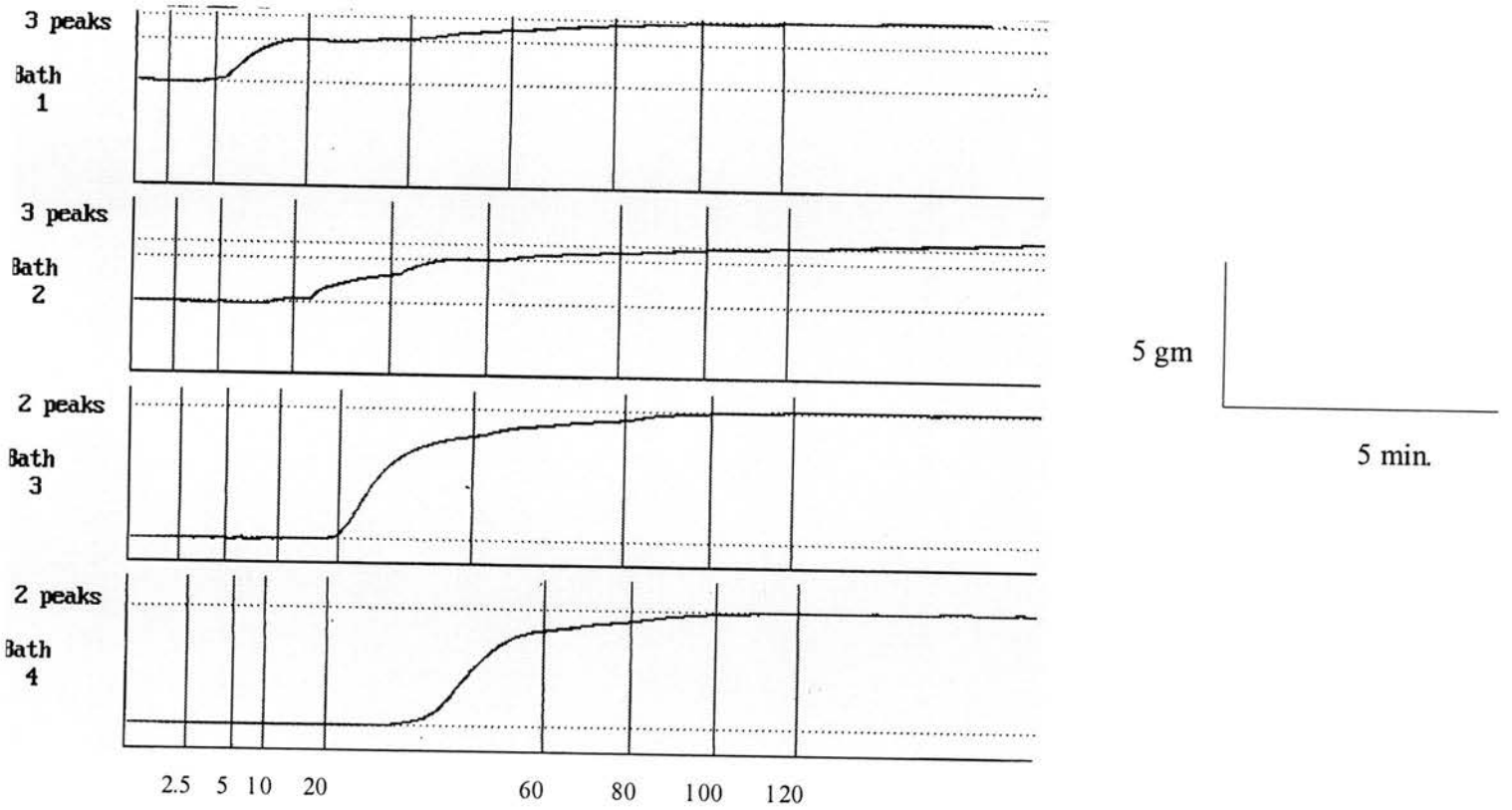
Appendix I: Sensitization of donor (Bath 1 & 2) and recipient (Bath 3 & 4) hepatic arteries with 100 mmol KCL solution showing reproducible contractions x 2 times.



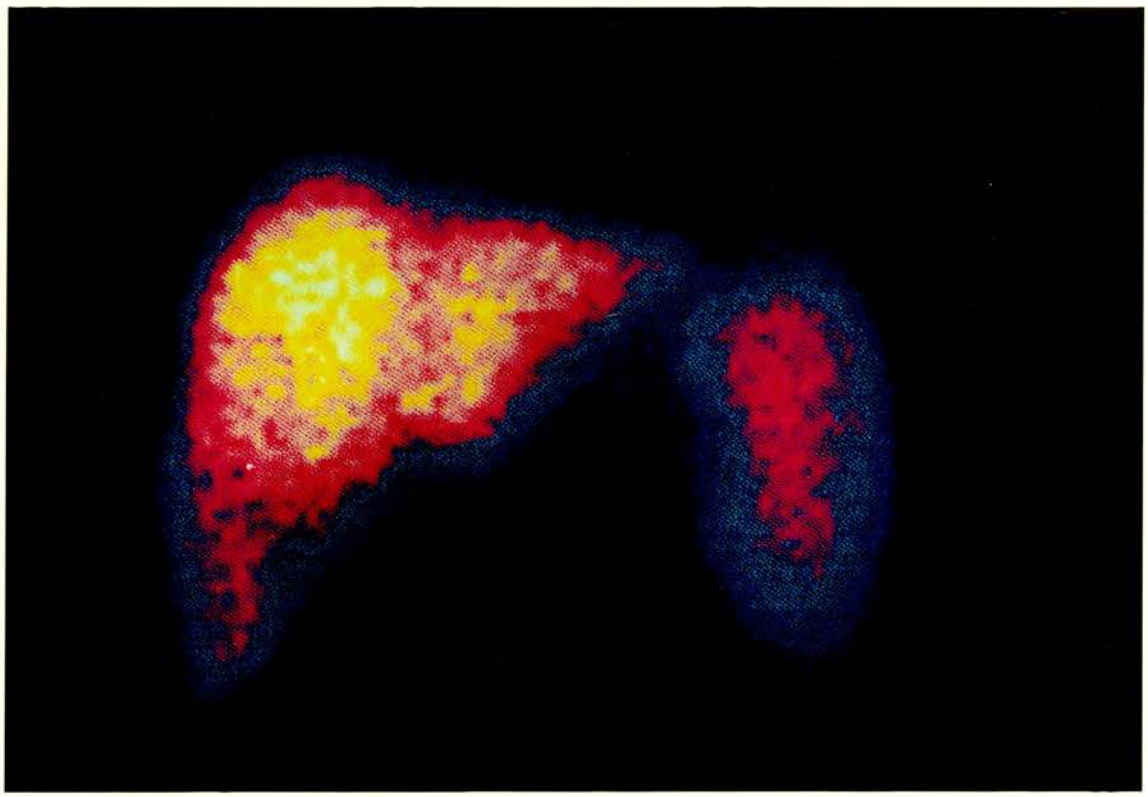
Appendix II: Incubation of donor hepatic arteries with D-NNA (Bath 1) and L-NNA (Bath 2) 10^{-4} M concentration, and recipient hepatic arteries incubation with D-NNA (Bath 3) and L-NNA (Bath 4) 10^{-4} M concentration.



Appendix III: Cumulative concentration response curve to PE in donor (Bath 1 & 2) and recipient hepatic arteries (Bath 3 & 4) incubated with D-NNA (Bath 1 & 3) and L-NNA (Bath 2& 4). Concentration of PE in Baths is expressed as M solution.

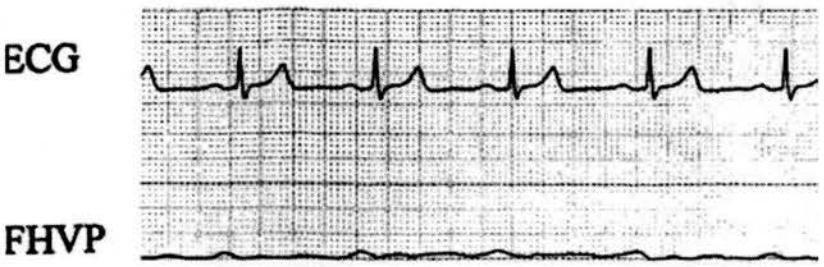


Appendix IV: Cumulative concentration curve to KCL (M solution) in donor (Bath 1 & 2) and recipient hepatic arteries (Bath 3 & 4).

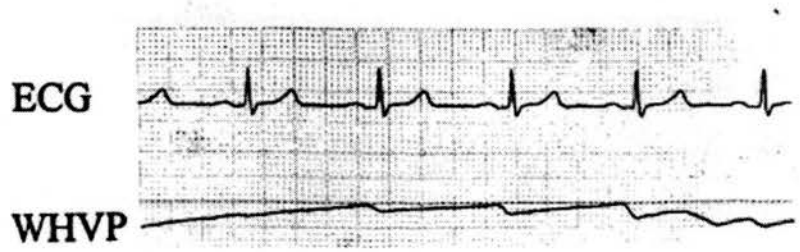


Appendix V: Hepato-splenic SPECT scan of one of the patients accomplished by administering $^{99}Tc^{m}$ albumin colloid.

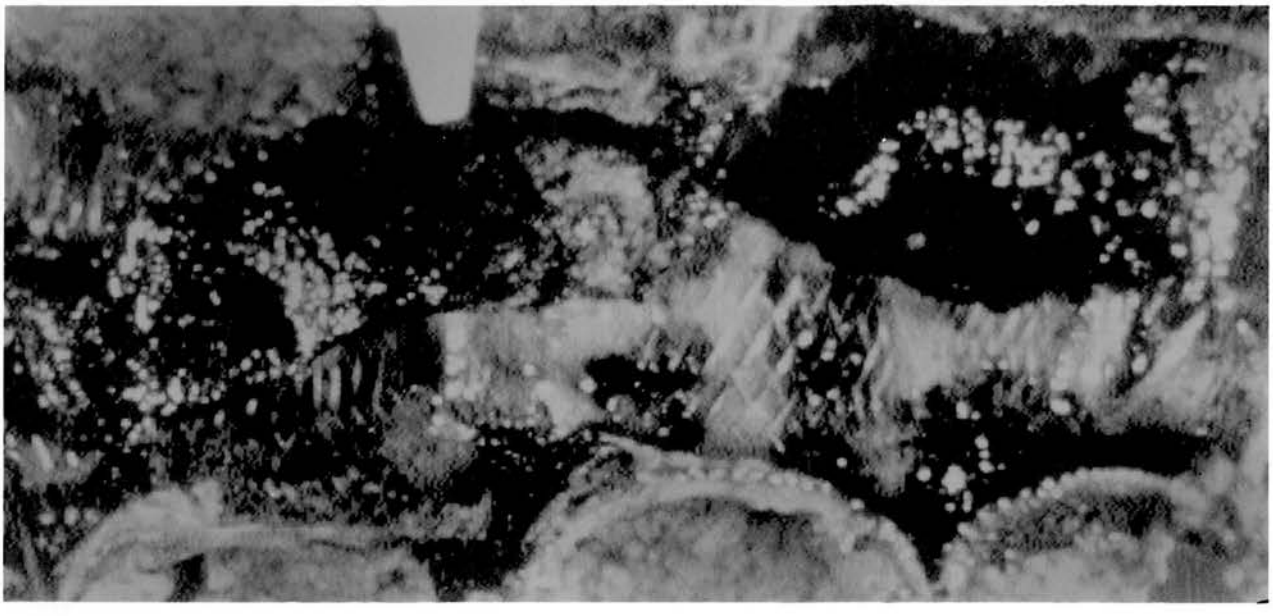
Free hepatic venous pressure trace



Wedged hepatic venous pressure trace

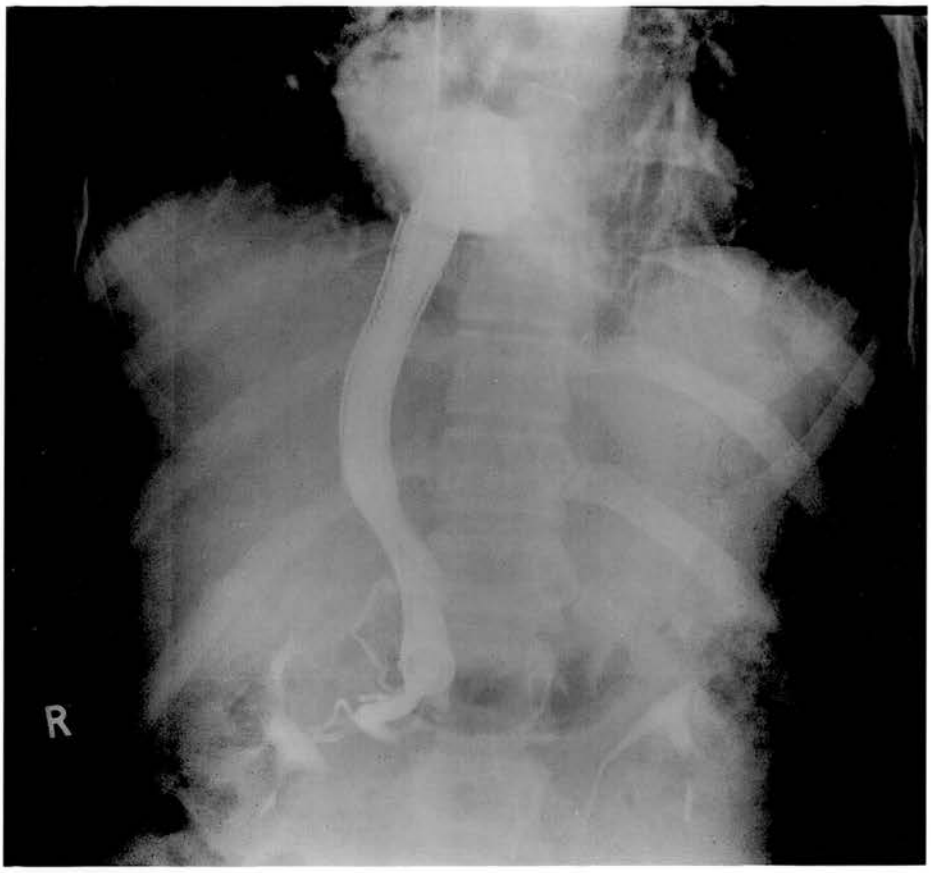


Appendix VI: Free and Wedged Hepatic Venous pressure trace

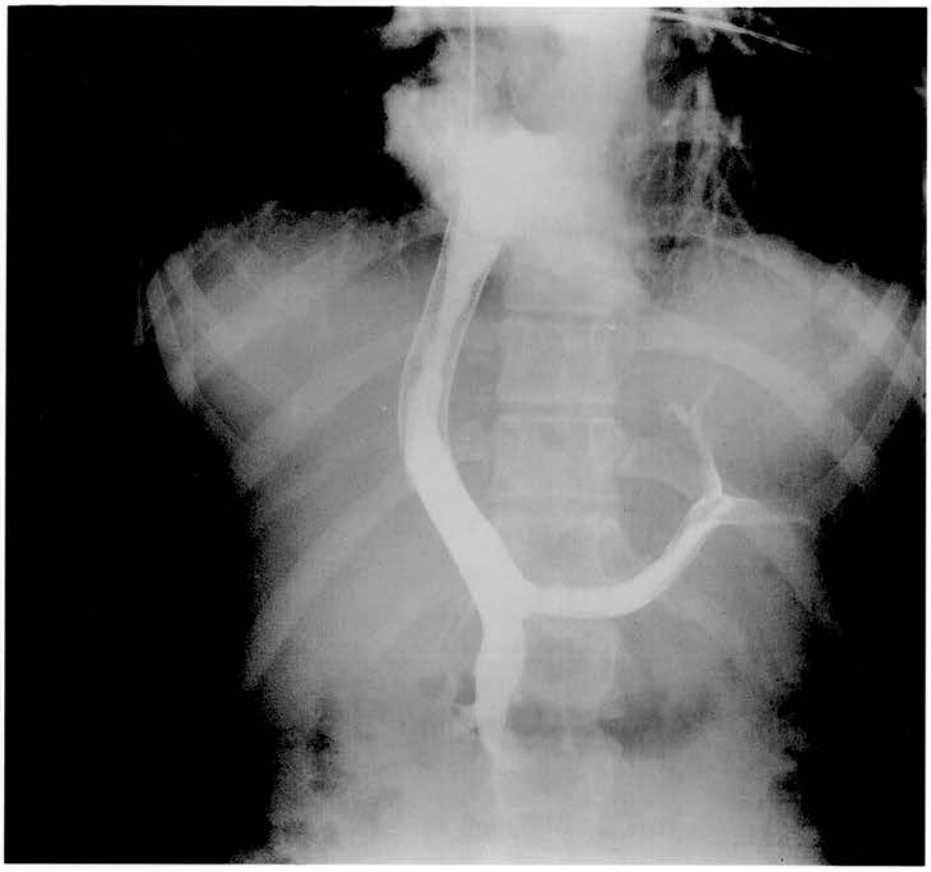


Appendix VII: Gross morphology of TIPSS, opened up after surgical removal of cirrhotic liver at OLT. It shows metal mesh with numerous blood clots with fibrin deposits.

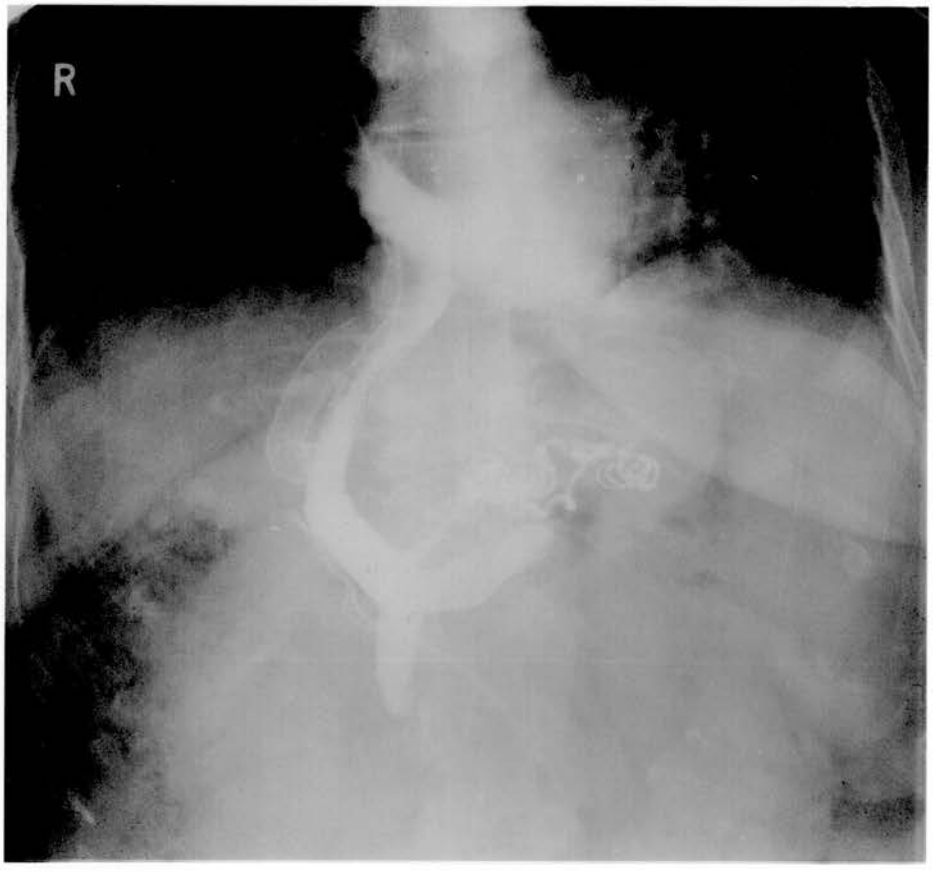
Appendix VIII: Histology of pseudo-intimal growth over TIPSS as shown on H&E staining. It comprises of endothelium and underlying supporting tissue.



Appendix IX: Radiograhic appearance of minimal pseudointimal hyperplasia at check portography.



Appendix X: Radiographic appearance of gross pseudointimal hyperplasia at portography.



Appendix XI: Radiographic appearance of pseudointimal hyperplasia in a parallel TIPSS placed after occlusion of first stent.

PUBLICATIONS

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Measurement of Spleen Size and Its Relation to Hypersplenism and Portal Hemodynamics in Portal Hypertension Due to Hepatic Cirrhosis

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Aims: **Splenomegaly is common in portal hypertension due to hepatic cirrhosis, but there are little data comparing different methods of spleen measurement. We have compared ultrasound with radionuclide imaging in measuring splenomegaly. The relation of splenomegaly to hypersplenism and portal hemodynamic factors was also studied.** ***Results:*** **Ultrasound and radionuclide measures of spleen volume gave comparable results ($r = 0.95, p < 0.0001$). Phagocytic activity of the spleen measured by radionuclide uptake increased as the volume of the spleen increased ($r = 0.46, p < 0.03$) but was not related to diminishing liver phagocytic activity. Spleen volume was correlated negatively with leukocyte counts ($r = 0.43, p < 0.05$) but not with hemoglobin or platelet counts. Spleen radionuclide uptake was negatively correlated with hemoglobin ($r = 0.48, p < 0.04$) and leukocyte counts ($r = 0.46, p < 0.04$) but not with platelet counts. Spleen volume was related to portal vein cross-sectional area ($r = 0.91, p < 0.0001$) and portal vein blood flow volume ($r = 0.57, p < 0.008$) but not to portal vein blood flow velocity, portal pressure gradient, or azygos blood flow.** ***Conclusions:*** **Spleen size measured by ultrasonography and radionuclide studies gives comparable results. Spleen phagocytic activity in cirrhosis increases as the spleen enlarges but not in relation to decreased hepatic phagocytic activity. Spleen phagocytic activity probably contributes to anemia and leukopenia in the splenomegaly of cirrhosis, but other factors must contribute to thrombocytopenia. Portal hemodynamics are probably important in the splenomegaly, but the interrelation is complex.**

INTRODUCTION

Splenomegaly and hypersplenism are common in portal hypertension due to cirrhosis, but the mechanisms whereby they occur continue to be debated. Attempts to analyze the relation of spleen size to factors such as portal pressure, portal hemodynamics, and hypersplenism require reliable methods of measuring spleen size. Methods used include abdominal radiography (1), ultrasonography (2, 3), radionuclide imaging (4–6), computed tomography (7), and magnetic resonance imaging. Few studies have investigated the comparability of these various methods (8), leading to uncertainty in relating studies using different methods to one another. Accordingly, we have compared ultrasound, which measures spleen volume, with radionuclide imaging, which measures uptake of colloid by the reticuloendothelial cells of the spleen, in measuring splenomegaly in patients with cirrhosis of liver and portal hypertension. We have also related the results to peripheral blood features of hypersplenism. Attempts to analyze the relation of spleen size to such factors as portal pressures and portal hemodynamics have seldom been done and relatively few patients have been studied (9–11). We have compared spleen volume with portal hemodynamic measurements to see whether these might explain the splenomegaly of portal hypertension.

PATIENTS AND METHODS

Twenty-five patients (sixteen males) with hepatic cirrhosis and portal hypertension were studied. Cirrhosis was diagnosed by liver biopsy in seven patients; of these seven patients, portal hypertension was diagnosed by wedged hepatic venous pressure measurement (WHVP) in three patients and by finding esophageal varices in four patients. Cirrhosis was diagnosed by clinical features, abnormal liver function tests, and ultrasonography in 18 patients: of these patients, portal hypertension was diagnosed by WHVP measurement in 9, from the presence of esophageal varices in 7, and splenomegaly in 2 patients. Of the 15 patients with alcoholic cirrhosis, 5 had primary biliary cirrhosis, 2 had autoimmune hepatitis and cirrhosis, 1 had hepatitis C virus liver disease, and 2 had cryptogenic cirrhosis.

Ultrasound imaging was carried out after an overnight fast using an Acuson 128 with a 3.5 MHz transducer. The abdomen was scanned initially and the shape, size, and texture of the liver was noted, together with the presence or absence of portasystemic collaterals and ascites. The spleen

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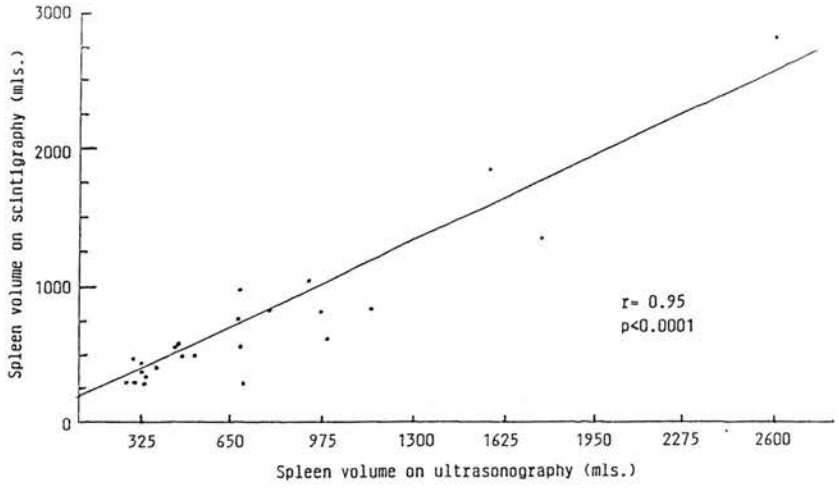

FIG. 1. Relation of spleen volume measured by ultrasonography to spleen volume measured by radionuclide imaging (scintigraphy).

was then examined. The greatest length, transverse diameter, and thickness at the hilum were measured. These were then multiplied together and a further factor of 0.6 was included to obtain an approximation of the volume. The portal vein was examined during quiet respiration with the patient supine and in the right anterior oblique position. The cross-sectional area was measured immediately below the bifurcation on three occasions and a mean value obtained. Mean averaged velocity was then measured using pulsed Doppler on three occasions, and a mean value was calculated. An approximation of the flow volume was then calculated by multiplying the average cross-sectional area (mm^2), the mean time averaged velocity (cm/sec), and a factor of 60 to obtain flow volume in ml/min.

Radionuclide imaging of liver and spleen was carried out after administration of 140 MBq of Tc 99m albumin colloid and single photon emission computed tomography (SPECT) was performed using a GE 400 AT gamma camera and a Siemens Microdelta Plecs Computer. Data were collected in 64 views, each 64 × 64 pixels; over an arc of 360 degrees. Transverse slices were constructed by back projection using a Butterworth filter of order 3 with a cut-off at 0.5 of the Nyquist frequency. Liver and spleen volumes were determined by a semi-automatic method whereby the operator viewed the transverse slices as a movie sequence, first determining which slices contained images of the organ concerned and then drawing a rectangular region of interest (ROI) that enclosed the entire organ. Each frame was then smoothed twice using a nine-point weighted filter to reduce statistical noise, and the volume of the cuboid described by the rectangular ROI and the selected frames were searched to determine the maximum value. The operator was shown each frame in turn and given the opportunity to redraw the ROI of the frame to exclude intruding structures (*eg*, liver in the spleen ROI). Then, for each frame, all pixels above a threshold of 50% of the maximum were summed. The 50% threshold was determined by calibrating the pixel size, then imaging phantoms with volumes in the range 400–1000 ml at various thresholds to determine which gave the most accurate result.

The WHVP and free hepatic vein pressure (FHVP) were measured at hepatic vein catheterization using a balloon occlusion sidewinder catheter (Cordis), and the portal pressure gradient (PPG) calculated (PPG = FHVP − WHVP). The azygos blood flow was measured by azygos vein catheterization using a reverse thermodilution catheter (Webster Laboratories) with real-time display using a dedicated computer as previously described (12).

RESULTS

There was a close correlation between ultrasonically and isotopically determined spleen volume (Fig. 1) ($r = 0.95$, $p < 0.001$). There was a good correlation between the spleen volume determined ultrasonically and portal vein cross-sectional area ($r = 0.91$, $p < 0.0001$) and portal vein blood flow volume (ml/min) ($r = 0.57$, $p < 0.008$) (Fig. 2, 3). No significant correlation was found among spleen size, the portal pressure gradient, the portal vein blood flow velocity, or the azygos blood flow. The radionuclide uptake of the spleen increased as the volume of the spleen increased ($r = 0.46$, $p < 0.03$), and there was no correlation between liver and spleen radionuclide uptake. Spleen volume was negatively correlated with the white blood cell count ($r = 0.43$, $p < 0.05$), but no correlation with hemoglobin or platelet count was observed. The phagocytic activity of the spleen as evidenced by radionuclide uptake was negatively correlated with hemoglobin ($r = 0.48$, $p < 0.04$) and white cell count ($r = 0.46$, $p < 0.04$), but no correlation was found between spleen radionuclide uptake and platelet count.

DISCUSSION

Splenomegaly is a cardinal feature of hepatic cirrhosis complicated by portal hypertension (13). The prevalence of

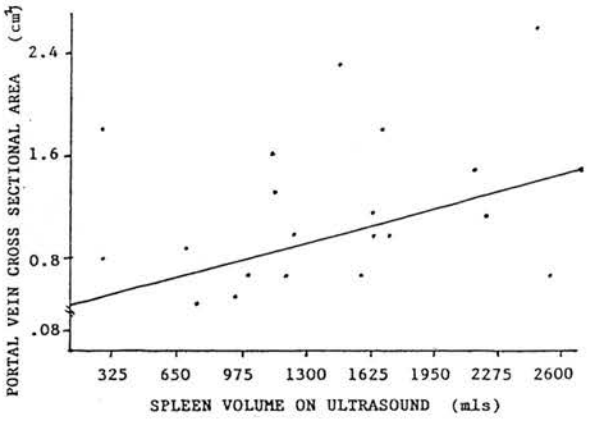

FIG. 2. Relation of spleen volume measured by ultrasonography to portal vein cross-sectional area. $r = 0.91$; $p < 0.0001$.

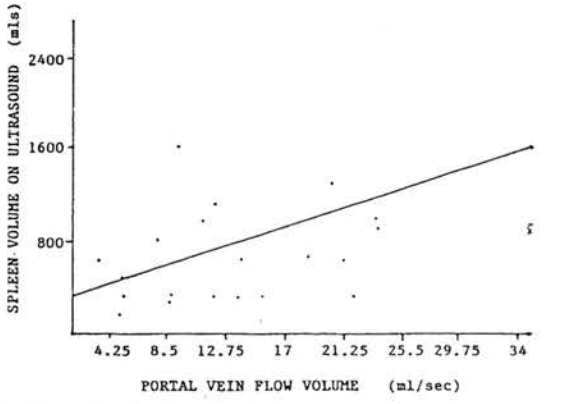

FIG. 3. Relation of spleen volume measured by ultrasonography to portal vein flow volume. $r = 0.57$; $p < 0.008$.

splenomegaly in cirrhosis varies from 36–92% (14). The cause of splenomegaly in cirrhosis and portal hypertension is not well understood, but may include reticuloendothelial hyperplasia and the hemodynamic changes associated with portal hypertension. Several methods have been used to visualize the spleen *in vivo*, and these include abdominal radiography (1), ultrasonography (2, 3), computed tomography (7), and radionuclide uptake methods (4–6). Few studies, however, have addressed the comparability of these methods of measuring spleen size (8). The close correlation of spleen volume as determined by ultrasonography and radionuclide studies in our patients with splenomegaly due to cirrhosis of liver suggests that studies using these methods are comparable, and perhaps other methods are also comparable. This finding is in agreement with findings of Zhang and Lewis (15) who compared methods of measuring spleen size in hematological disorders.

A number of factors could be important in the production of hypersplenism in splenomegaly. Hypersplenism can be regarded as the association of one or more of anemia, leukopenia, and thrombocytopenia with splenomegaly, and a normal or hypercellular bone marrow. The formed elements of blood are suppressed to varying degrees with a recovery after splenectomy (16). Various mechanisms to account for the development of hypersplenism in the splenomegaly of portal hypertension in cirrhosis have been proposed (17). These include increased pooling of blood cells in the spleen, increased destruction of blood cells in the spleen, the dilutional effects of an increased blood volume, and of humoral factors (18). Thrombocytopenia is the most common manifestation of hypersplenism in cirrhosis and portal hypertension, and previous studies have reported a negative correlation between the platelet count and spleen size (19). We, however, could not find any correlation between the platelet count and ultrasonic spleen volume or the phagocytic activity of the spleen as reflected in the radionuclide scan suggesting that other factors must also be important. These could include the presence and amount of immunoglobulin on the platelet surface (20). Our results showed a negative correlation of the white cell count to spleen size measured by ultrasonography and radionuclide imaging, and spleen size may therefore be more important in relation to leukopenia in hypersplenism. The increased reticuloendothelial phagocyte activity noted on radionuclide imaging may well be important in relation to the white blood cells. The hemoglobin was inversely related to the radionuclide ultrasonography. Phagocytosis may therefore be more important in relation to red blood cells.

Ultrasonography measures total spleen mass by assessing volume, whereas scintigraphy depends upon functionally active reticuloendothelial tissue to show splenic dimensions. We found good comparability of these two methods of measuring spleen size; this finding means that phagocytic cell mass in the enlarged spleen increases proportionately to its total volume as is seen in the splenomegaly of hematological disorders (15). Thus, reticuloendothelial cell hyperplasia might be important in producing splenomegaly. The increase of phagocytic activity is not a consequence of diminishing liver phagocytic activity because there was no correlation between liver and spleen colloid uptake on scintigraphic measurement in our patients. This conclusion is not in agreement with the results of previous studies (21).

Splenomegaly in cirrhosis is accompanied by important portal hemodynamic changes. Splenomegaly in cirrhotic patients may in fact result from these pathophysiological changes (22, 23). The strong positive correlation of portal vein cross-sectional area and portal blood flow volumes with spleen size emphasizes the importance of circulatory factors in splenomegaly, but does not indicate whether this is a cause or a result of splenomegaly. Furthermore, the lack of correlation of spleen size with portal vein pressures and collateral blood flow through the azygos vein implies a complex relationship. One interpretation of these hemodynamic data is that spleen size is determined, at least early in the disease process, by portal congestion, thereby explaining the close association with portal vein diameter. As liver disease progresses, congestion does not increase because decompression via portal collaterals, measured by azygos

flow occurs. Indeed, portasystemic shunting through the azygos system increases as liver disease advances (24). The lack of correlation with portal pressure may reflect individual differences in compliance of the portal vascular bed and portasystemic shunting to changes in portal pressure. It is interesting that although a number of studies have found Doppler ultrasound measurements valuable in predicting events or drug responses in patients with portal hypertension, it is the congestion index (ratio of portal vein cross-sectional area and velocity) rather than portal vein cross-sectional area alone that is clinically relevant, emphasizing again the complex nature of the relationship (25).

There may be a temporal separation between the development of established portal pressure and the mechanisms causing splenomegaly. Our patients had established portal hypertension, often with esophageal varices, which are a later development in the condition, and hemodynamic measurements at this stage may not reflect changes present during the development of splenomegaly. In conclusion, our results point generally to the importance of circulatory factors in splenomegaly but cannot delineate precise mechanisms.

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Direct Measurement of Pulsatile Insulin Secretion from the Portal Vein in Human Subjects*

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ABSTRACT

Insulin is secreted in a high frequency pulsatile manner. These pulses are delivered directly into the portal vein and then undergo extraction and dilution before delivery into the systemic circulation. The reported frequency of these insulin pulses estimated in peripheral blood varies from an interpulse interval of 4–20 min. We postulated that this discrepancy is due to the attenuation of the pulse signal in the systemic circulation *vs.* the portal circulation. In the present study we measured pulsatile insulin release directly in the portal circulation of human subjects who had indwelling transjugular intrahepatic portasystemic stent shunts (TIPSS) to decompress portal hypertension. We quantitated pulsatile insulin secretion in both the overnight fasted state (fasting) and during a hyperglycemic clamp (8 mmol/L). Direct portal vein sampling established that pulsatile insulin secretion in humans has an interval (periodicity) of approximately 5 min. The amplitude (and mass) of the insulin concentration oscillations observed in the portal vein was approximately 5-fold greater than that observed in the arterialized vein and was similar to that observed in the dog. Increased insulin release during hyperglycemia was achieved through amplification of the insulin pulse mass. In conclusion, direct portal vein sampling in humans revealed that the interpulse interval of insulin pulses in humans is about 5 min, and this frequency is also observed when sampling from the systemic circulation using a highly specific insulin assay and 1-min sampling, but is about 4-fold greater than the frequency observed at this site using single site RIAs. We confirm that enhanced insulin release in response to hyperglycemia is achieved by amplification of these high frequency pulses. (*J Clin Endocrinol Metab* **85:** 4491–4499, 2000)

INSULIN IS SECRETED in high frequency pulses (1). This pattern of insulin secretion has been shown to be abnormal in subjects with type 2 diabetes (2) and their first degree relatives (3) and in patients at risk of developing type 1 diabetes (4). However, quantification of pulsatile insulin secretion is complex. Insulin pulses are secreted into the portal circulation and undergo significant hepatic extraction and waveform damping before entering the systemic circulation (5). We previously developed a canine model of direct portal vein catheterization to overcome this problem (6) and reported that sampling from the systemic circulation resulted in an underestimate of both the frequency of insulin pulses as well as the calculated proportion of insulin released in the pulsatile mode (5). Inevitably, studies of pulsatile secretion in humans have been confined to the systemic circulation (2–4, 7–12). Initially such studies reported a pulse frequency of 15–20 min (2–4, 7, 8), but more recently a pulse frequency of about 6 min has been suggested (11, 12). We have speculated that these differences may reflect the greater sensitivity of novel insulin assays applied in the latter studies, and that the true frequency of insulin pulses delivered into the portal circulation in humans may be 6–8 min. The latter would be comparable to the frequency observed in the isolated perfused pancreas (13) as well as the perifused islets (14, 15).

In the current study we applied a validated deconvolution technique for quantifying pulsatile insulin secretion to plasma insulin concentration profiles obtained simultaneously from the portal vein and the systemic circulation in human subjects with stable compensated hepatic cirrhosis and an *in situ* transjugular intrahepatic portasystemic stent shunt (TIPSS) (16). The TIPSS catheter allowed relatively noninvasive high frequency sampling of blood from the portal vein in conscious human subjects. Pulsatile insulin secretion was quantified in the fasting basal state as well as during a hyperglycemic clamp study.

Using this protocol we sought to address the following questions. First, is the frequency of pulsatile insulin secretion in humans (observed by direct sampling from the portal circulation) comparable to the frequency reported previously from studies in isolated islets, the isolated pancreas, and *in vivo* by sampling from the portal vein in dogs (interpulse interval, ~6–8 min or ~15–20 min as reported in some studies using the systemic sampling site in humans). Second, does hyperglycemia in the human (as in the dog) enhance insulin secretion through the specific mechanism of augmenting the mass of insulin bursts? Finally, we examined whether the amplitude of the insulin concentration wavefront to which the liver is exposed in humans approaches that observed in the portal vein of dogs (5, 6).

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Subjects and Methods

Study subjects and design (Table 1)

This study was approved by the Lothian ethics committee (institutional review board equivalent), and all volunteers provided informed written consent. Inclusion criteria were patients with a patent TIPSS and known stable compensated liver disease. Patients were deliberately selected to represent those in which the predominant liver-related problem was cirrhosis and portal vein hypertension treated with a TIPSS, rather than progressive liver failure or active hepatic inflammation. Exclusion criteria include decompensated liver dysfunction, diabetes mellitus, a thrombosed stent, and/or a prolonged prothrombin time. All patients were studied 1–2 weeks before the TIPSS study and had a 75-g oral glucose tolerance test performed to exclude diabetes mellitus. Of six subjects screened, one was excluded from the study because of diabetes.

Five patients with *in situ* TIPSS were studied immediately after a routine follow-up assessment of the patency of their TIPSS. For this purpose patients were admitted overnight into the Royal Infirmary of Edinburgh. After an overnight fast a catheter was placed in the right jugular vein, and under ultrasound guidance it was passed through the TIPSS into the portal circulation. The purpose of the clinical assessment was to ensure that the TIPSS remained patent. Once this had been confirmed, a catheter was inserted via the right internal jugular introducer sheath into the portal vein to allow the present study to be completed.

Study protocol

All study subjects were admitted to the University of Edinburgh Wellcome Trust Clinical Research Facility at the Royal Infirmary of Edinburgh the night before the study and remained fasting overnight. On the morning of the study, each subject underwent TIPSS portogram performed by a radiologist as part of the routine follow-up assessment of the patency of the *in situ* TIPSS, and the sampling catheter was placed in the portal vein at the end of this procedure.

A peripheral indwelling sampling catheter was also inserted into a dorsal hand vein and the hand was warmed to 40 C by an electric blanket to permit subsequent sampling of arterialized blood. An iv catheter was also placed in an antecubital vein in the contralateral arm and infused with saline at 30 mL/h. Once all of the catheters were in place there was a 45-min rest period before commencement of the protocol at approximately 1000 h. At protocol time 0–40 min, simultaneous intensive 1-min sampling of arterialized blood and portal vein blood was performed to obtain the minutely insulin concentration profile at each site in the fasting state. At protocol time 40 min, a hyperglycemic clamp was commenced with the object of raising the arterialized plasma glucose concentration to 8–9 mmol/L. This was achieved by infusion of a variable rate glucose infusion (50% dextrose) administered by a programmable infusion pump (Harvard Infusion Pumps, Ayer, MA) on-line to a personal computer. Arterialized blood was sampled at 5-min intervals, and plasma glucose was measured within 2 min. Steady state hyperglycemia was achieved by protocol time 80 min (40 min after the clamp was begun), and then the second sampling period began. From protocol time 80–120 min, blood was sampled at 1-min intervals from the arterialized peripheral vein catheter and the portal vein catheter to determine the insulin concentration profile during the hyperglycemic clamp. Portal vein sampling was completed in all five study subjects in both the basal and hyperglycemic sampling periods. Arterialized sampling was completed in four of the five subjects; the peripheral sampling catheter was unreliable in the remaining subject. In two subjects portal vein blood flow was measured during the study by use of Doppler scanning.

TABLE 1. Patient characteristics

Case no.	Age (yr)	Sex	Wt (kg)	BMI (kg/m^2)	FPG (mmol/L)
1	58	F	60	23	4.4
2	61	M	71	22	5
3	43	M	71	24	4
4	61	M	100	33	5.8
5	54	M	117	36	5.4

FPG, Fasting plasma glucose; M, male; F, female; BMI, body mass index.

Assays

Plasma glucose concentrations were measured by the glucose oxidase method using a glucose analyzer (Beckman Coulter, Inc., Palo Alto, CA).

Plasma insulin concentrations were measured in duplicate by two-site immunospecific insulin enzyme-linked immunosorbent assay (ELISA), as previously described (11, 17). In brief, the assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmark) specific for human insulin. The detection range of this insulin ELISA was 5–600 pmol/L. At medium (150 pmol/L), medium-high (200 pmol/L), and high (350 pmol/L) plasma insulin concentrations, the interassay coefficients of variation were 3.7%, 4.0%, and 4.5%, and the corresponding intraassay variations were 2.3%, 2.1%, and 2.0%. There was no cross-reactivity with proinsulin or split 32,33 and des-31,32 proinsulins, respectively.

The plasma C peptide concentration was assayed with a commercially available kit (K6218, DAKO Corp., Cambridgeshire, UK). The assay is a two-site ELISA based on two monoclonal antibodies, using the principles referred to above. Each sample was assayed in duplicate; intra- and interassay coefficients of variation were 2.2% and 3.3%, respectively.

Data analysis: detection and quantification of pulsatile insulin secretion by deconvolution analysis

The plasma insulin concentration-time series were analyzed by deconvolution as previously validated (18) to detect and quantify insulin secretory bursts (6). Deconvolution of plasma insulin concentration data

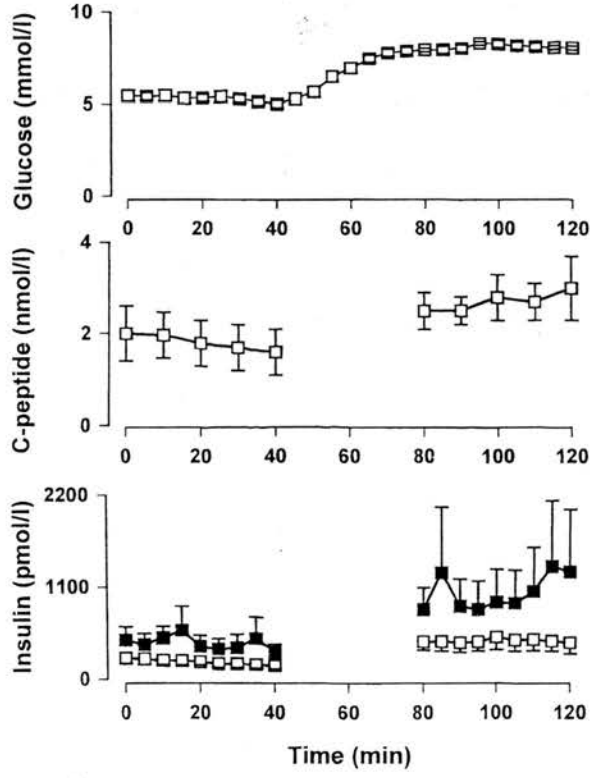

FIG. 1. Mean arterialized plasma glucose (*top panel*), C peptide (*middle panel*), and both arterialized (□) and portal vein (■) insulin concentrations. The time 0–40 min corresponds to the basal intensive sampling period. The hyperglycemic clamp was initiated at 40 min, and steady state hyperglycemia was achieved by 80 min, when the second intensive sampling period was undertaken.

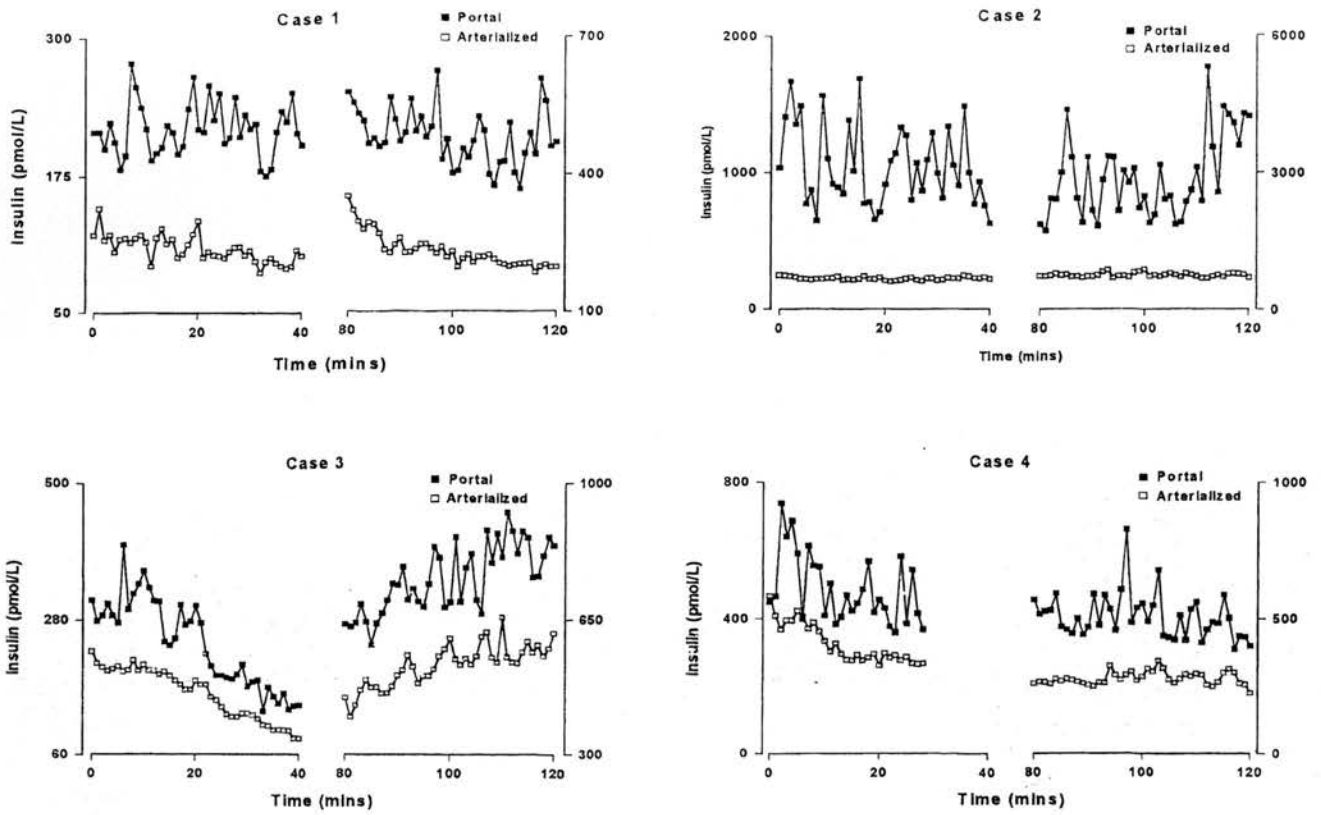

FIG. 2. Plasma insulin concentration profiles from the basal (0–40 min) and hyperglycemic clamp (80–120 min) periods in four cases obtained from the arterialized (□) and portal vein (■) sampling sites. Note that the scales are adjusted in the *left* and *right panels* to accommodate the insulin concentration range observed. In all cases, oscillations in insulin concentrations are much greater in the portal vein than in the arterialized sampling site.

was performed with a multiparameter technique that requires the following assumptions. Plasma insulin concentrations measured in samples collected at 1-min intervals were assumed to result from five determinable and correlated parameters: 1) a finite number of discrete insulin secretory bursts occurring at either regular or randomly dispersed times and having a) individual amplitudes (maximal rate of secretion attained within a burst) and mass (integral of the calculated secretory event) and b) a common half-duration (duration of an algebraically Gaussian secretory pulse at half-maximal amplitude), which are superimposed on c) a basal time-invariant insulin secretory rate; 2) a biexponential insulin disappearance model in the systemic circulation, consisting of earlier directly estimated half-lives of 2.8 and 5.0 min and a fractional slow component of 0.28 in healthy fasting humans; and 3) a biexponential insulin disappearance model in the portal circulation, consisting of half-lives of 1.0 and 3.0 min and a fraction slow component of 0.667. These parameters achieved the statistically best fit (maximally reduced fitted variance) of the portal vein insulin concentration profile. All secretory rates were expressed as mass units of insulin (picomoles) released per unit distribution volume (liters) per unit time (minutes). As the volume of distribution of insulin is unknown in the portal sampling site in humans and undoubtedly differs from that in the systemic circulation, insulin secretion rates obtained at these two sampling sites cannot be directly compared.

Statistical analysis

Data are presented as the mean ± SEM. Statistical comparison between groups was made using Student's two-tailed *t* test. To examine the relationship between insulin concentration fluctuations observed in the portal vein and systemic circulation, cross-correlation was per-

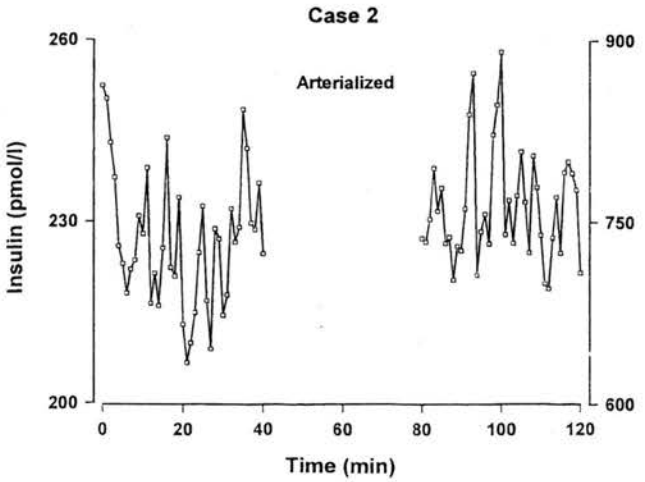

FIG. 3. Plasma insulin concentration profile observed from the arterialized sampling catheter in case 2 in the basal state (0–40 min) and during hyperglycemia (80–120 min). Note that the scale has been adjusted for each sampling period to maximize the visualization of oscillations. In comparison with the arterialized insulin concentration profiles in Fig. 2, the expanded scale clearly illustrates the prominent oscillations in insulin concentration in the both basal and stimulated states.

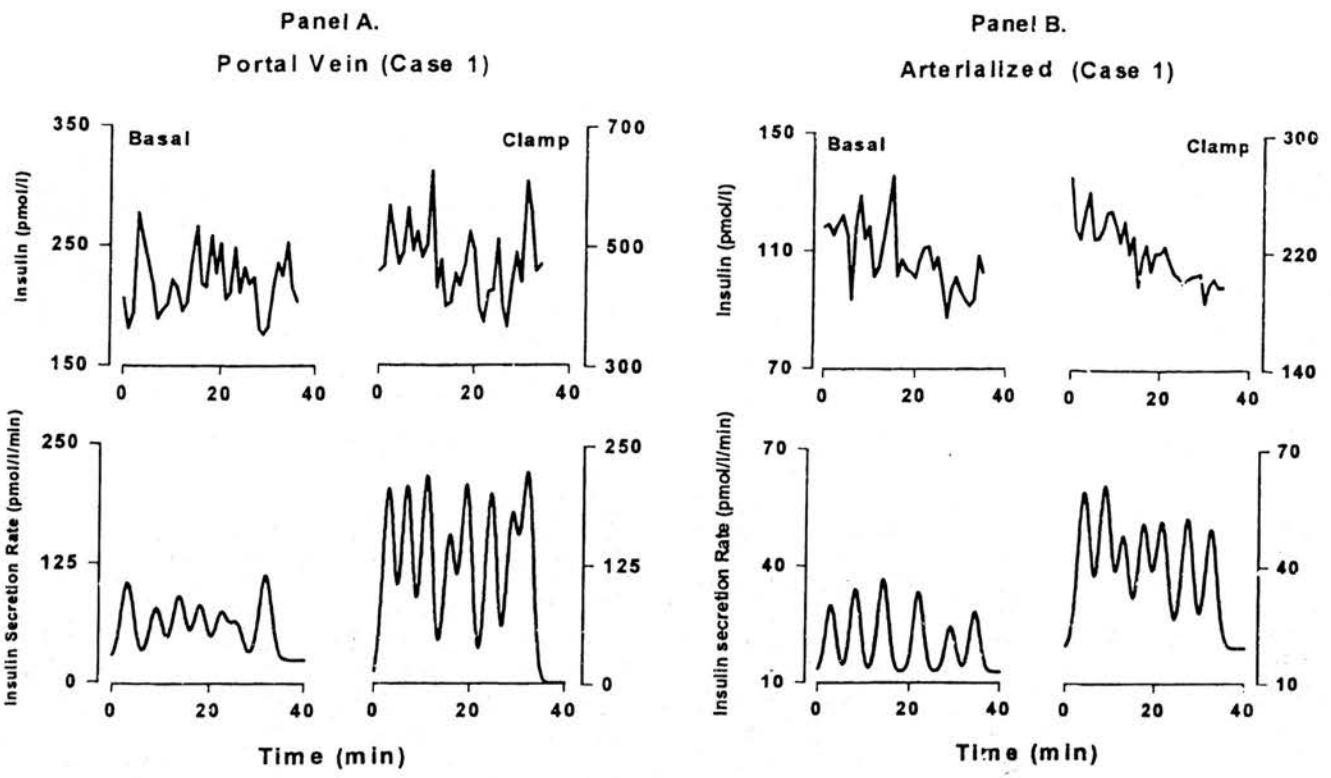

FIG. 4. A, The portal vein insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal state (*left panels*) and during the hyperglycemic clamp periods (*right panels*) for case 1. B, The arterialized insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal state (*left panels*) and during the hyperglycemic clamp periods (*right panels*) for case 1. Note that the insulin secretion rate is (mass units per volume of distribution)/time units. As the volume of distribution is unknown in the portal vein, these secretion units cannot be directly compared with those obtained from peripheral sampling.

formed (19). Cross-correlation analysis relates each portal vein insulin concentration to a corresponding value in the matching arterial series. This procedure consists of linear correlations carried out repeatedly at various time lags between the paired concentrations. Thus, at zero time lag, each portal vein plasma insulin concentration is compared with a time delayed measure (*e.g.* lag time minus 2 min) in the systemic circulation sample. By this means, an array of correlations can be collected that depend on the time matching of the two series.

To examine the relationship between pulses identified in the portal vein and systemic circulation we also performed peak concordance on these pulses from both sites. Peak concordance is a statistically independent (from cross-correlation) method to establish the relationship between detected pulses in two sampling sites. After identifying discrete insulin secretory bursts in the two time series (portal and peripheral), exact coincidence was defined by simultaneous pulse concordance (*i.e.* peak maxima occurred within one half-sampling interval of each other). Lagged coincidence was defined accordingly [*e.g.* with portal (+lags) or peripheral (−lags] peaks occurring first. The hypergeometric probability density (joint binomial distribution) was used to estimate the expected number of randomly concordant pulses, and the probability of falsely refuting the null hypothesis of pure chance concordance of the observed coincidences.

Results

Mean plasma glucose, insulin, and C peptide concentrations (Fig. 1)

The mean arterialized plasma glucose concentrations during the basal (fasting) and stimulated (hyperglycemic clamp) sampling periods were 5.3 ± 0.3 and 8.1 ± 0.1 mmol/L, respectively ($P < 0.001$). As expected, there was a rise in the mean arterialized (basal *vs.* stimulated, 209 ± 7.4 *vs.* 456 ± 16.8 pmol/L, $p < 0.001$) and mean portal vein (basal *vs.* stimulated, 440 ± 25.3 *vs.* 1020.7 ± 72.3 pmol/L; $P < 0.001$) insulin concentration after the increase in the plasma glucose concentration from 5.3 to 8 mmol/L during the hyperglycemic clamp. The mean arterialized C peptide concentration also increased with hyperglycemia (basal *vs.* stimulated, 1.8 ± 0.1 *vs.* 2.7 ± 0.1 nmol/L; $P < 0.001$), confirming an increase in insulin secretion in response to the glucose stimulus. Throughout the study in each subject, the portal vein insulin concentration was higher than the corresponding arterialized insulin concentration in both basal ($P < 0.001$) and stimulated ($P < 0.001$) sampling periods (Fig. 1).

Portal vein blood flow

The mean portal vein blood flow values in the two cases in which it was measured were 1.1 and 0.8 L/min. There was no change in the portal vein blood flow between the basal state and the hyperglycemic clamp.

Insulin concentration profiles (Figs. 2 and 3)

Inspection of the plasma insulin concentration profiles from the individual patients indicated the presence of re-

TABLE 2. Liver function studies

Type of cirrhosis	Alk phos (40–125 U/L)	GGT (5–35 u/L)	Bilirubin (2–17 μmol/L)	Albumin (36–47 g/L)	ALT (10–40 μL)	INR (2–4.5)
Alcoholic	125	124	56	32	14	1.4
Alcoholic	80	20	17	43	27	1
Alcoholic	146	100	42	34	56	1.3
Alcoholic	135	61	147	35	35	1.7
Alcoholic	92	54	17	41	33	1.2

ALT, Alanine amino transferase; Alk Phos, alkaline phosphatase; GGT, γ-glutamyl trasferase; INR, international normalized ratio. Normal ranges in *parentheses*.

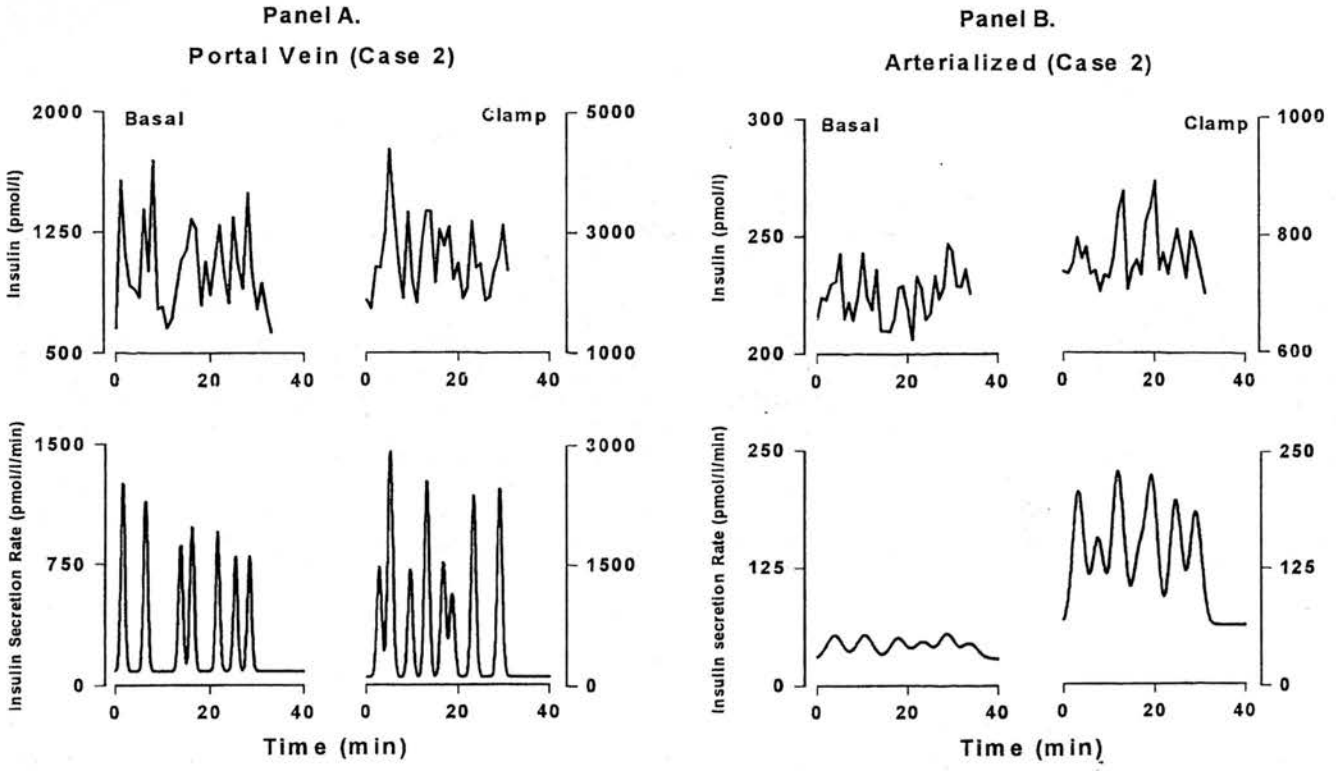

FIG. 5. A, The portal vein concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal state (*left panels*) and during the hyperglycemic clamp periods (*right panels*) for case 2. B, The arterialized insulin concentration profile and corresponding deconvolved insulin secretion rates obtained during the intensive sampling periods in the basal state (*left panels*) and during the hyperglycemic clamp periods (*right panels*) for case 2.

current oscillations in both arterial and portal circulations in all patients. The magnitude of these oscillations was much larger in the portal circulation (Fig. 2) in both the basal and stimulated states compared with the corresponding profiles from the systemic circulation. The amplitude of the oscillations increased during hyperglycemia (Fig. 2). The range of the insulin pulse amplitude observed in the portal circulation was 100-1000 pmol/L in the basal state and increased to 200-3000 pmol/L during the hyperglycemic clamp. This contrasts with the corresponding range of pulse amplitudes observed in the systemic circulation of 10–30 and 40–100 pmol/L in the basal and stimulated states, respectively (Fig. 3).

Pulse detection

When the insulin concentration profiles were subjected to deconvolution, insulin pulses were invariably identified in both the portal and systemic circulation (Figs. 4 and 5). The pulse mass increased in response to hyperglycemia in both portal (basal *vs.* stimulated, 418 ± 155 *vs.* 1078 ± 368 pmol/L; $P < 0.05$) and systemic (basal *vs.* stimulated, 75 ± 10 *vs.* 241 ± 61 pmol/L; $P < 0.05$) circulations (Fig. 6). Furthermore, the measured pulse mass/volume of distribution was about 5-fold larger in the portal circulation in both basal and stimulated states. The pulse amplitude determined by deconvolution was also markedly (7- to 8-fold) larger in the portal circulation in both basal (portal *vs.* systemic, 254 ± 158 *vs.* 23 ± 2.7 pmol/L·min) and stimulated (portal *vs.* systemic, 524 ± 337 *vs.* 72 ± 19.8 pmol/L·min) states, confirming the impression gained by inspection of the insulin concentration profiles. The corresponding four pulse half-duration estimates were similar, with a global mean of 2.9 min. The interpulse interval (Fig. 6) determined by direct portal vein sampling was similar in the basal sampling period and during the clamp (Fig. 6). The pulse interval detected by sam-

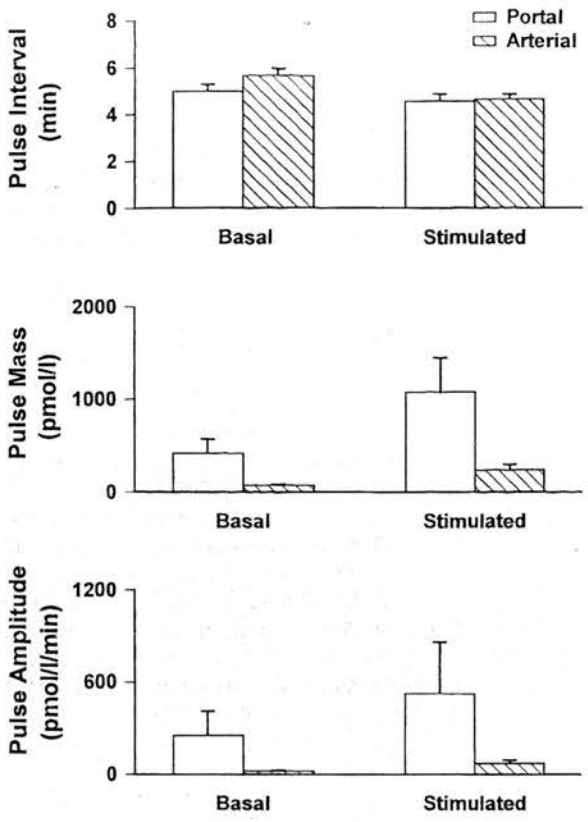

FIG. 6. The mean insulin interpulse interval (*top panel*), pulse mass (*middle panel*), and pulse amplitude (*bottom panel*) during the basal sampling period and the stimulated hyperglycemic period evaluated by sampling from the portal vein (*open panels*) *vs.* the arterial catheter (*shaded panels*). Units are minutes (pulse interval), picomoles per L (mass), and picomoles per L/min (amplitude).

pling from the systemic circulation was slightly, but not significantly, higher than that observed in the portal circulation.

In the portal circulation, the calculated proportion of insulin derived from discrete insulin secretory bursts was 64 ± 1.6% in the basal state and 93 ± 2.9% during the hyperglycemic clamp. In contrast, in sampling from the systemic circulation, the calculated proportion of insulin secreted in the pulsatile mode was apparently lower than that from the simultaneous sample measurements obtained from the portal circulation (basal state, portal *vs.* systemic, 66 ± 1.8% *vs.* 35 ± 4.1%; $P < 0.05$) and stimulated (portal *vs.* systemic, 93 ± 2.9% *vs.* 56 ± 1.2%; $P < 0.01$). Both autocorrelation and peak concordant analysis independently revealed a relationship between insulin oscillations in the portal vein and the systemic circulation (Figs. 7 and 8).

Discussion

The present study uses direct and high frequency sampling from the portal vein to affirm that insulin is secreted in humans in discrete secretory bursts, and that hyperglycemia enhances insulin secretion by selective amplification of the mass of insulin contained within each secretory burst. Furthermore, we could establish that the prehepatic frequency of insulin pulses is approximately 5 min, which, in fact, corresponds to that observed in the portal vein of dogs *in vivo* (5, 6) and from isolated perfused islets *in vitro* (14, 15).

Our conclusion that most insulin secreted into the portal circulation is derived from pulses is contingent upon the assumptions required for the deconvolution method employed (see *Materials and Methods*). It should also be noted that the half-life for insulin used in these studies for portal vein insulin concentration deconvolution was obtained by a best-fit approach to the portal vein insulin concentration profiles. Direct measurement of the volume of distribution or half-life at this site by a bolus injection upstream of the TIPSS sampling catheter in a mesenteric vein is not practicable in conscious humans. Nonetheless, similar studies in dogs with surgically implanted catheters arrived at the same conclusion that most insulin is secreted in discrete insulin bursts (6). Also, a prior study in humans sampling from the systemic circulation came to the same conclusion (11).

There has been some disagreement in the literature about the frequency of pulsatile insulin secretion *in vivo*, with estimates ranging from about 6 (5, 6) to about 20 (2–4, 7, 8) min. When we measured pulsatile insulin release directly from the canine portal vein, the interpulse interval averaged 6 min, based on a conventional insulin immunoassay and a deconvolution program specifically validated for insulin pulse detection (5, 6). Although this pulse frequency corresponded to that observed in perfusion of single islets *in vitro*, it was 3 times greater than that observed previously in humans by sampling from the systemic circulation (2–4, 7, 8). We postulated that the reason for this large discrepancy was the relative loss of insulin signal when insulin pulses are examined in the systemic compared with the portal circulation (6).

At least in the canine model, we confirmed that the sampling site was crucial when a conventional insulin assay was employed (5), as simultaneous estimates in the portal vein and the systemic circulation revealed insulin interpulse intervals of 6.7 and 9 min, respectively (5). The insulin pulse detection was further obscured by using a 2-min sampling regimen in the systemic circulation, as employed in some previous reports (7). The disadvantages of sampling from the systemic circulation were especially evident when the only available insulin assays were conventional immunoassays (20). Even multiple replicates by these assays, which have cross-reactivity with proinsulin and insulin split products, do not allow for fully reliable detection of high frequency insulin pulses in the systemic circulation. However, the introduction of the more sensitive and specific ELISA assays for insulin (17) should theoretically overcome some of these problems. Recently, using an ELISA for insulin measurements, we reported an insulin pulse frequency of approximately 8 min when sampling in the systemic circulation in humans (11, 12). The present study now confirms directly that the systemic sampling route can provide an accurate estimate of insulin pulse frequency *in vivo* in humans when the insulin concentration is measured by a sensitive and specific ELISA method and submitted to appropriate deconvolution-based analysis.

Sampling directly from the portal circulation revealed a

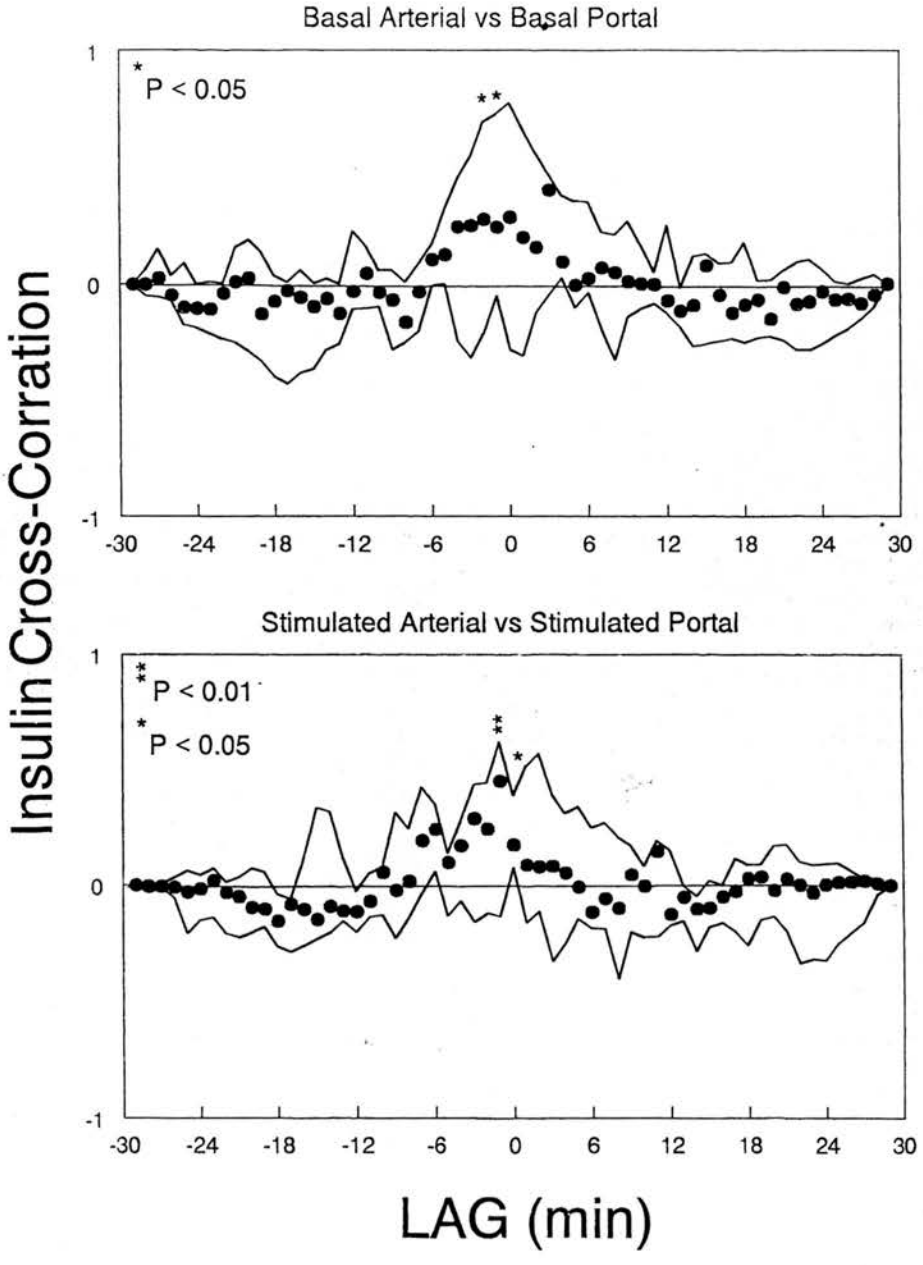

FIG. 7. Cross-correlation analysis of arterial and portal vein insulin concentration data in the basal and stimulated states. These data illustrate that changes in serial portal vein insulin concentrations precede those in the systemic circulation by 1–3 min ($P < 0.05$ and $P < 0.01$).

remarkable magnitude of insulin oscillations at this site. In the present study the amplitude of insulin concentrations in the portal vein was about 200-1000 pmol/L in the basal state and increased to about 500-5000 pmol/L during hyperglycemia. These values in the human are comparable with those observed in the canine portal vein (5, 6) and presumably reflect the insulin concentration wavefront to which the liver is exposed. Although the present study subjects were necessarily only those with portal hypertension requiring treatment, we selected subjects with normal or near-normal liver function tests who were clinically well and had normal prestudy OGTTs. Moreover, the range of insulin oscillations observed in the portal vein in these subjects is comparable to that previously observed in healthy dogs (5, 6), and the portal vein blood flow, when available, was in the normal reported range for humans. Although some studies have reported that pulsatile insulin delivery (to the peripheral circulation) enhances insulin sensitivity, none has yet recapitulated the magnitude of insulin oscillations to which the liver is exposed *in vivo* during stimulated enhanced insulin secretion (21, 22). The question remains, therefore, what the physiological importance is, if any, of exposing the liver to such dramatic oscillations of insulin concentration. As the magnitude of peripheral insulin pulses is decreased in patients

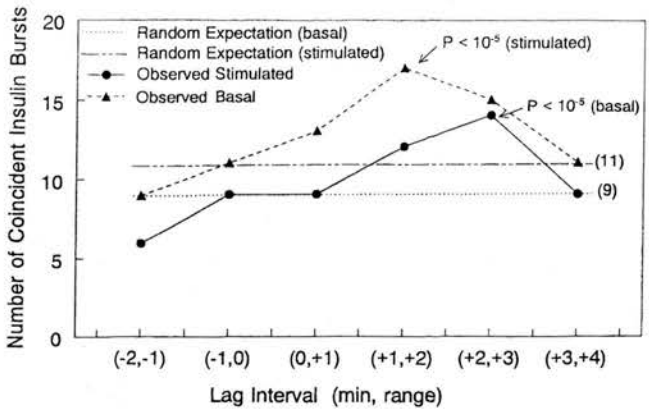

FIG. 8. Peak concordance analysis of pulses detected in the portal vein and systemic circulation. Observed number of coincident insulin secretory bursts detected by simultaneous sampling in the portal and peripheral circulation. The *x*-axis gives the lag interval between the individually identified portal and peripheral insulin pulses. A lag interval of (−2, −1) denotes that pulses occurred centered within this absolute time lag (*i.e.* the pulses in the portal blood followed those of insulin in the peripheral blood by no less than 1 min and no more than 2 min). Thus, at a lag interval of (+1, +2), insulin pulses in the portal blood preceded those in the peripheral blood by no less than 1 min and no more than 2 min (*right side* of graph). The expected number of purely random coincidences was calculated via the hypergeometric probability distribution in both the basal and stimulated states (*interrupted lines*). The observed number of coincidences significantly exceeded expectation values at 1- to 3-min lag times, wherein portal insulin pulses appeared first. The maximal coincidence was 13 events in the basal state, and 17 events in the stimulated state. The corresponding expected values were 9 and 11 given the different pulse frequencies at these times, with respective P values less than 10^{-5}.

with type 2 diabetes (23), it is likely that the liver in these patients is exposed to even more attenuated oscillations in insulin pulse amplitude, which may contribute to the hepatic insulin resistance characteristic of this disease (24).

The remarkable transhepatic attenuation of the insulin pulse signal from the portal circulation to the systemic circulation observed here is comparable to that observed previously in the dog (5). Although the attenuation may reflect up to an approximately 4-fold dilution of portal venous blood by the systemic venous drainage, the insulin pulse amplitude is damped to a much greater extent than this (~10-fold). One potential explanation for this is preferential hepatic insulin clearance of insulin pulses. We previously noted that the hepatic clearance of portal vein insulin in the dog is related in an ascending monotonic (quadratic) manner to the pulse amplitude of the insulin concentration wavefront presented to the liver (25). Taken together, these preliminary observations provoke the need for additional studies to address this possibility of a link between pulse waveform and hepatic insulin clearance. In one prior study in which blood was sampled from the portal vein of patients with cirrhosis, comparable large oscillations in insulin concentration were observed, although no quantification of pulsatile insulin release or corresponding arterial sampling was available (26).

In summary, direct sampling from the portal vein in humans establishes an insulin interpulse interval of about 5 min and thereby resolves the discrepant insulin pulse frequency previously reported in humans. A comparable insulin interpulse interval can also be detected by high frequency peripheral sampling with the use of a highly sensitive insulin assay and appropriately validated pulse detection. Direct portal vein sampling is consistent with prior studies in dogs and humans, which reported that the majority of insulin is secreted in insulin bursts. Finally, we confirm that the liver in humans, like that in dogs, is exposed to dramatic insulin concentration oscillations that far exceed the magnitude evaluated to date in clinical studies of insulin action *in vivo*.

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Transjugular intrahepatic portosystemic stent-shunt insufficiency and the role of diabetes mellitus

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Background/aims **Maintenance of long-term patency of transjugular intrahepatic portosystemic stent-shunts (TIPSS) has proved problematic. Various prognostic variables have been assessed as predictors, but the role of diabetes mellitus, which induces vascular endothelial cell dysfunction, has not been assessed.**

Methods **We analysed the records of 248 patients who underwent TIPSS between July 1991 and July 1997, followed-up through to August 1998. Patients with at least one shunt assessment by portography and available blood glucose levels were eligible (177 patients; median follow-up, 15.0 months). Fourteen patients had a pre-procedural diagnosis of diabetes (one insulin dependent, seven oral hypoglycaemic treated and six diet controlled). In another 14 patients, diabetes was diagnosed at TIPSS insertion, giving a 28/177 (15.8%) prevalence of diabetes in our patients. Fifty-nine patients were excluded from the final analysis (including five diabetics), as they either died or had early shunt insufficiency (within 1 month of stent placement), leaving 118 patients (including 23 diabetics) to be included in the final analysis.**

Results **Mean age, sex distribution, median follow-up (months) and pre-shunt portal pressure gradient were comparable in the two groups (diabetics versus non-diabetics). Child–Pugh classes A and B were more common in the diabetic group ($P < 0.01$), and the mean inserted stent diameter was larger in the diabetic group ($P < 0.05$). The presence of diabetes was associated with a higher incidence of delayed shunt insufficiency ($P = 0.02$), but there was no evidence of an association between presence of diabetes and variceal haemorrhage post TIPSS. Kaplan–Meier analyses revealed earlier insufficiency in diabetic patients compared with those without diabetes ($P = 0.04$). Age, gender and presence of diabetes are included in the final logistic regression model. Individuals who have diabetes are more likely to experience shunt insufficiency independent of age and gender.**

Conclusions **Diabetes mellitus is common in patients undergoing TIPSS and is associated independently with increased incidence of primary delayed shunt insufficiency.** *Eur J Gastroenterol Hepatol* **13:257–261 © 2001 Lippincott Williams & Wilkins**

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Keywords: diabetes mellitus, shunt insufficiency, transjugular intrahepatic portosystemic stent-shunt

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Introduction

Transjugular intrahepatic portosystemic stent-shunt (TIPSS) is an effective method of treating portal hypertension and its complications [1]. However, shunt insufficiency and occlusion are major limiting factors in the success of this form of treatment [2]. Primary shunt patency at 6 months, and 1 and 2 years has been reported as 71.2, 58.2 and 21.4%, respectively [3]. Rates of shunt insufficiency in other studies have been shown to vary from 25 to 80% [4–9]. Shunt stenosis can usually be treated by balloon angioplasty, extension of the stent or the insertion of a co-axial stent, but reocclusion occurs [10,11].

The pathogenesis of shunt stenosis is not entirely clear. One of the factors determining this is the time course of occlusion. While early occlusion is associated with thrombosis, delayed shunt dysfunction is predominantly due to pseudo-intimal hyperplasia within the shunt [12]. A number of factors have been assessed as predictors of shunt insufficiency [13] and biliary venous leak in the shunt lumen is also implicated [10]. Diabetes mellitus is common in patients with cirrhosis of the liver [14]. It leads to smooth muscle cell proliferation and endothelial cell dysfunction in blood vascular wall, especially the systemic arterial bed [15]. Similar mechanisms might be operative in the portal

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vascular bed in portal hypertension and thus may be responsible for delayed shunt insufficiency due to pseudo-intimal hyperplasia in patients who have had TIPSS.

Patients and methods

The cohort of patients ($n = 248$) who underwent TIPSS insertion between July 1991 and July 1997 was reviewed, looking for shunt dysfunction post-TIPSS and its relation to diabetes mellitus.

Patients were included in the analysis if TIPSS insertion was successful, at least one portographic shunt assessment was completed post-insertion, blood sugar profile was available for a diagnosis of diabetes mellitus to be made and early TIPSS insufficiency (within 1 month post-TIPSS) was absent. Patients were not entered into the analysis if they had incomplete data, portographic follow-up was not available, early TIPSS insufficiency was present or hepatocellular carcinoma was present.

One hundred and seventy-seven patients (71.4%) had successful TIPSS placement, and had blood sugar profiles available for a diagnosis of diabetes mellitus to be made or excluded up to the time of TIPSS insertion. Fifty-nine patients were excluded because of either early death, early shunt insufficiency, referral for portosystemic shunt surgery or liver transplantation within the first month following TIPSS insertion. The clinical data of the 118 patients who were entered into the final analysis comparing diabetic versus non-diabetic patients are presented in Table 1. TIPSS insertion was undertaken according to the method described elsewhere [6]. The procedure was carried out under sedation and analgesia with local anaesthesia, unless general anaesthesia was indicated because of the clinical condition of the patient. Patients were followed-up by Doppler ultrasound at day 7 post shunt placement to exclude early thrombosis. Regular follow-up by portography was undertaken at 6-monthly intervals unless shunt occlusion was clinically suspected, which led to earlier portographic shunt assessment.

Data were censored when the patient: (1) died, (2) had documented shunt insufficiency, (3) had liver transplantation, or (4) portographic surveillance was discontinued.

Definitions

Primary shunt insufficiency (PSI) was defined as the increase in portal pressure gradient (PPG) to > 12 mmHg [16] or an increase in portal pressure greater than 20% from baseline, when the baseline post-TIPSS portal pressure was above 12 mmHg [3,6].

PSI was further classified into early (< 1 month post shunt placement) and delayed (> 1 month post shunt placement). Diabetes was either diagnosed on oral glucose tolerance test or on the basis of two random blood glucose levels > 11 mmol/l taken 6 months apart (World Health Organization criteria). Rebleeding was defined as overt haematemesis and/or melaena, or a drop of haemoglobin of greater than 2 g/dl without any other demonstrable source of blood loss or haemolysis. Endoscopy confirmed gastro-oesophageal variceal bleed or portal hypertensive gastropathy.

Statistical analysis

The statistical analysis was carried out using the Statistical Package for Social Scientists software (SPSS Inc. Chicago, Illinois). In order to compare risk factors for shunt insufficiency in the diabetic and non-diabetic patients, univariate testing was first carried out. *t*-Tests were used to compare mean age, pre-shunt PPG and post-shunt PPG, in the two groups of patients. Non-parametric Mann–Whitney tests were performed to compare length of follow-up and shunt-diameter in the two groups, and chi-square tests were performed to test for associations between presence of diabetes, gender, Child's class, primary delayed insufficiency and variceal haemorrhage.

In order to determine which factors were independently associated with primary delayed insufficiency, logistic regression modelling was carried out. Several clinical and biological variables were considered for

Table 1 Comparison of clinical data and shunt characteristics in diabetic versus non-diabetic patients ($n = 118$) undergoing TIPSS with no demonstrable early shunt insufficiency

Factor	Diabetic ($n = 23$)	Non-diabetic ($n = 95$)	*P* value
Age in years (mean ± SD)	57.3 ± 11.2	52.2 ± 12.5	0.07
Gender (males/females)	18/5	57/38	0.15
Child's Class (A + B/C)	20/3	54/41	0.01
Follow-up (months) (median; IQR)	15.0, 22.0	17.2, 18.2	0.43
Pre-shunt PPG (mmHg) (mean ± SD)	19.6 ± 5.5	19.2 ± 7.0	0.79
Post-shunt PPG (mmHg) (mean ± SD)	8.5 ± 4.0	6.8 ± 4.6	0.11
Stent diameter (mm) (median; lower quartile, upper quartile)	12.0; 12.0, 14.0	12.0; 10.0, 12.0	0.02
Stent diameter (mm) (mean ± SEM)	12.2 ± 0.32	11.44 ± 0.16	0.04

SD, standard deviation; IQR, interquartile range; SEM, standard error of the mean.

inclusion in the model, including age, gender, Child's class, shunt diameter, pre- and post-shunt PPG, and whether the patient had diabetes. The final logistic regression model includes a subset of variables that are significantly associated with delayed PSI, independent of the other factors also included in the model. Kaplan–Meier survival analysis was used to compare time to delayed shunt insufficiency and a log-rank test was applied to check for statistical significance of the difference.

Results

Diabetes was present in 28 out of 177 (15.8%) patients who had both successful TIPSS and blood sugar profiles. Of the 118 patients who met inclusion criteria, 23 (19.5%) patients were diabetic and 95 (80.5%) non-diabetic. Statistical testing provided no evidence that the diabetic patients and non-diabetic patients differed in age, sex, pre-shunt PPG, post-shunt PPG and length of follow-up (Table 1). However, the presence of diabetes was found to be associated with a better Child's class ($P = 0.01$); diabetic patients were more likely to have a Child's class A or B (87.0%) than non-diabetics (56.8%). The median inserted shunt diameters were equal in the two groups, but the interquartile ranges showed diabetics to have larger shunts in place ($P = 0.02$).

A comparison of clinical and demographic features between the study sample and the entire TIPSS treated population is presented in Table 2 and confirms the representative nature of the sample in comparison with the TIPSS patient population from which it is derived.

The frequency of delayed PSI in diabetic versus non-diabetic patients is presented in Table 3. Diabetic patients were more likely to suffer from primary delayed insufficiency (87.0%) than non-diabetic patients (60.0%). This is unlikely to be due to a difference in Child's classes among the two groups as the incidence of shunt insufficiency is similar in Child's classes A and B compared with C (Table 4). There was no evidence of an association between the presence of diabetes and variceal haemorrhage post shunt insertion. Cumulative rate of diagnosed shunt patency is presented in Figure 1, which shows significantly reduced patency rates over time in diabetic patients with cirrhosis of the liver ($P = 0.04$).

Table 5 presents odds ratios (ORs) and 95% confidence intervals (CIs) for the factors included in the final logistic regression model. These factors are statistically significant when included in the model, and are independently associated with delayed shunt insufficiency. Females are more likely than males, and diabetics are more likely than non-diabetics, to experience shunt insufficiency (OR = 3.44, 95% CI = 1.37–8.61; OR = 7.30, 95% CI = 1.87–28.43, respectively). However, individuals aged 55 years or over are less likely than those in the younger age group to experience shunt insufficiency (OR = 0.40, 95% CI = 0.17–0.94).

Table 2 Comparison of clinical data and shunt characteristics of the study sample versus the whole group of TIPSS treated patients[a]

Factor	Study sample ($n = 118$)	Total TIPSS treated patients ($n = 248$)
Age in years (mean ± SD)	53.4 ± 12.7	53.7 ± 12.9
Gender		
Male	73	156
Female	45	92
Indication for TIPSS		
Oesophageal varices	75	155
Gastric varices	19	40
Ascites	16	36
PHG	3	6
Oesophageal + rectal varices	3	8
Hydrothorax	2	3
Aetiology of liver disease		
Alcoholic cirrhosis	81	161
Cryptogenic cirrhosis	5	21
PBC	10	19
Post-hepatitic cirrhosis	12	20
Congenital fibrosis	3	6
PSC	3	5
CAH	3	6
Miscellaneous	1	10
Procedure		
Elective	45	141
Emergency	73	107
Child–Pugh classes		
A + B	65	115
C	53	133

[a]Independent samples *t*-test and chi-square test were applied to test for association between the two groups and the *P* value was found to be not significant in comparing different factors.
SD, standard deviation; PHG, portal hypertensive gastropathy; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; CAH, chronic active hepatitis.

Discussion

This study included a large cohort of patients with TIPSS who underwent long-term portographic follow-up. Diabetes has been shown to be associated with increased prevalence of stent occlusion following coronary balloon angioplasty and stent implantation [16–20]. If restenosis is viewed as an intraluminal growth process after vascular injury, a similar mechanism might be operative in TIPSS occlusion as in coronary stent occlusion. Although there are haemodynamic differences between the high-pressure arterial circulation in the coronary circulation and the low-pressure portal venous circulation, the turbulent flow in the TIPSS, especially at the hepatic venous end of the stent (where pseudointimal hyperplasia is most marked), makes it liable to injury from stress of the jet stream of blood. The process of repair and endothelial regeneration,

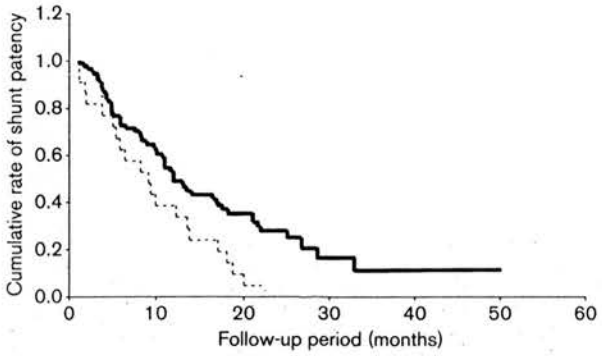
Table 3 Outcomes in diabetic versus non-diabetic patients followed-up for primary delayed insufficiency

Factor	Diabetic ($n = 23$)	Non-diabetic ($n = 95$)	P value
Primary delayed insufficiency	20 (87.0%)	57 (60.0%)	0.02
Variceal haemorrhage	4 (17.4%)	12 (12.6%)	0.52
Post-TIPSS hepatic encephalopathy	4 (17.4%)	12 (12.6%)	0.52

Table 4 Primary delayed shunt insufficiency and variceal haemorrhage in relation to Child's class of liver disease

Outcome	Child's Class A + B ($n = 71$)	Child's Class C ($n = 47$)	P value
Primary delayed insufficiency	51 (71.8%)	26 (55.3%)	0.16
Variceal haemorrhage	11 (15.5%)	5 (10.6%)	0.78

Fig. 1



Cumulative rate of primary shunt patency compared in diabetic (dotted line) versus non-diabetic (solid line) cirrhotics ($P = 0.04$).

Table 5 Final logistic regression model of primary delayed shunt insufficiency

Factor	Number of subjects	Odds ratio (95% CI)	P value
Diabetes			
Non-diabetic	95	1.00	< 0.01
Diabetic	23	7.30 (1.87–28.43)	
Gender			
Male	75	1.00	< 0.01
Female	43	3.44 (1.37–8.61)	
Age			
< 55 years	61	1.00	0.04
> 55 years	57	0.40 (0.17–0.94)	

which may result in pseudointimal hyperplasia and shunt insufficiency, follows this.

In our study population, the diabetic and non-diabetic groups were comparable in their clinical characteristics except for Child's class and stent diameter. Child's class was better and larger stents were used in the diabetic group. Child's class, however, was not associated with increased prevalence of delayed PSI (Table 4), and larger diameter stents in diabetic cirrhotics would have made them less liable to occlude. Furthermore, all factors in Table 1 were considered for inclusion in the logistic regression model, and Child's class and shunt diameter were not independent risk factors.

Table 2 demonstrates that, although the study sample is only $n = 118$ (47%) of the entire TIPSS treated group, it is still comparable with and representative of the latter in its clinical and demographic features. The number of patients who underwent placement of TIPSS as an emergency and those with Child's grades A and B were proportionately slightly greater in the study sample as compared with that in the entire TIPSS treated population, but that did not achieve statistical significance. Although different complications of portal hypertension from liver diseases of varied aetiology led to treatment with TIPSS, the most commonly treated entity in our centre was bleeding oesophageal varices due to alcoholic cirrhosis.

The lack of evidence of an association between variceal haemorrhage post shunt occlusion and the presence of diabetes is surprising but may be due to the close follow-up in our patients that allowed early intervention. Some of the patients also underwent endoscopic band ligation after TIPSS, which would reduce the risk of bleeding.

On post-TIPSS follow-up, hepatic encephalopathy developed for the first time in 4/23 (17.4%) diabetic cirrhotics (Table 3) and needed standard therapeutic measures to be instituted. Although this was a higher incidence than in the non-diabetic patients, it was not statistically significant. The precipitation of encephalopathy did not follow episodes of gastro-intestinal bleeding and two patients each had Child's grades B and C, respectively.

This is the first study looking at the role of diabetes mellitus, and occlusion and insufficiency of TIPSS. Our results indicate that the presence of diabetes is associated with delayed shunt insufficiency, independent of age and gender. Further work needs to be carried out to determine if this may be a possible causal effect. Hyperglycaemia induces alterations of growth factor secretion [15], which may be important in the genesis

of shunt narrowing due to fibrous tissue and endothelial overgrowth. The role of factors such as insulin-like growth factor (IGF-1), transforming growth factor beta (TGF-β) and endothelins (which have increased expression and secretory rates in individuals with diabetes and hyperglycaemia [21–23]) in smooth muscle cell replication and pseudo-intimal hyperplasia in the shunt lumen should be investigated. From a practical standpoint, the results of this study would suggest that patients should be screened for diabetes at shunt implantation and a meticulous glycaemic control maintained post-TIPSS to prevent hyperglycaemia, and therefore hopefully minimize delayed shunt insufficiency.

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NOS INHIBITION DOES NOT ALTER ADRENOCEPTOR-MEDIATED CONTRACTION OF HUMAN HEPATIC ARTERIES, *IN VITRO*.

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Background: Generation of nitric oxide (NO) by the inducible form of NO synthase (iNOS) in vascular smooth muscle cells may contribute to impaired contractile function in patients with cirrhosis. This remains controversial, however, as studies of isometric contraction in arteries from patients and animals with cirrhosis have produced conflicting results.

Aims: This investigation aimed to determine whether the release of NO from vascular smooth muscle cells modulates α-adrenoceptor-mediated contraction of hepatic arteries from patients with cirrhosis.

Methods: Hepatic arteries were obtained from recipients (cirrhotic; n=9) and donors (non-cirrhotic; n=8) during orthotopic liver transplantation for *in vitro* measurement of isometric contraction. Two rings, 2mm in length, were taken from each artery and suspended in parallel in modified Krebs'-Henseleit solution at 37°C, perfused with 95% O_2; 5% CO_2. Rubbing the luminal surface of each vessel ensured removal of the endothelium. One ring from each artery was incubated with the NOS inhibitor N^G-nitro-L-arginine (L-NNA; 10^{-4}M) whilst the other was incubated with its inactive isomer(D-NNA;10^{-4}M). Cumulative concentration-response curves were obtained using phenylephrine (PhE; 10^{-9}-$3x10^{-4}$M) and KCl (2.5-120 mM).

Results: PhE (and KCl; data not shown) produced contractions of similar size and sensitivity (pD_2) in arteries from cirrhotic (9.33±1.09g and 5.26±0.22, respectively) and non-cirrhotic (10.46±1.71g and 5.67±0.31 respectively) subjects. Incubation of vessels with L-NNA did not alter the response of either donor or recipient arteries to PhE (Table1).

Table 1.

	Recipient Hepatic Artery (n=9)			Donor Hepatic Artery (n=8)		
	D-NNA	L-NNA	*P*	D-NNA	L-NNA	*P*
M.C. (g)	9.3±1.1	11.5±1.2	0.21	10.5±1.7	11.2±1.8	0.75
M.C.(%KCl)	120.4±11.2	122.6±15.7	0.77	161.4±19.6	167.0±21.2	0.83
pD_2	5.3±0.2	5.6±0.2	0.25	5.7±0.3	5.6±0.3	0.86

Conclusions: These results indicate that the release of NO from the smooth muscle cells of the hepatic artery does not modulate α-adrenoreceptor-mediated contraction in patients with cirrhosis.

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